The Journal of Biological Chemistry

Volume 19

1914

Reprinted by arrangement with the American Society of Biological Chemists, Inc.

JOHNSON REPRINT CORPORATION
New York, New York

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

EDITED BY

. D. DAKIN, New York City. LAFAYETTE B. MENDEL, New Haven, Conn.
K. DUNHAM, New York City. A. N. RICHARDS, Philadelphia, Pa.
DONALD D. VAN SLYKE, New York City.

WITH THE COLLABORATION OF

J. J. ABEL, Baltimore, Md.
H. CHITTENDEN, New Haven, Conn.
'O FOLIN, Boston, Mass.
LLIAM J. GIES, New York.
L. J. HENDERSON, Cambridge, Mass.
REID HUNT, Boston, Mass.
W. A. JACOBS, New York.
WALTER JONES, Baltimore, Md.
J. H. KASTLE, Lexington, Ky.
J. B. LEATHES, Toronto, Canada.
P. A. LEVENE, New York.
JACQUES LOEB, New York.
A. S. LOEVENHART, Madison, Wis.
GRAHAM LUSK, New York.
A. B. MACALLUM, Toronto, Canada.
J. J. R. MACLEOD, Cleveland, Ohio.
JOHN A. MANDEL, New York.
A. P. MATHEWS, Chicago, Ill.
F. G. NOVY, Ann Arbor, Mich.
THOMAS B. OSBORNE, New Haven, Conn.
T. BRAILSFORD ROBERTSON, Berkeley, Cal.
P. A. SHAFFER, St. Louis, Mo.
A. E. TAYLOR, Philadelphia, Pa.
F. P. UNDERHILL, New Haven, Conn.
V. C. VAUGHAN, Ann Arbor, Mich.
ALFRED J. WAKEMAN, New Haven, Conn.

VOLUME XIX
BALTIMORE
1914

COPYRIGHT 1914
BY
THE JOURNAL OF BIOLOGICAL CHEMISTRY

CONTENTS OF VOLUME XIX.

W. R. Bloor: Studies on blood fat. I. Variations in the fat content of the blood under approximately normal conditions..... 1

Carl O. Johns and Byron M. Hendrix: Researches on purines. XV. On 2-oxy-5-amino-6-ethylaminopyrimidine and 2,8-dioxy-9-ethylpurine..... 25

Franklin C. McLean and Laurence Selling: Urea and total non-protein nitrogen in normal human blood: Relation of their concentration to rate of elimination..... 31

Frank P. Underhill and Norman R. Blatherwick: The elimination of phenolsulphonephthalein in acute and chronic tartrate nephritis..... 39

H. Gideon Wells and George T. Caldwell: The inhibition of autolysis by alcohol..... 57

Alfred W. Bosworth and Lucius L. Van Slyke: The phosphorus content of casein..... 67

Lucius L. Van Slyke and Alfred W. Bosworth: The cause of acidity of fresh milk of cows and a method for the determination of acidity..... 73

Carl L. Alsberg: Note on the proteins of the blood of *Limulus polyphemus* L..... 77

Samuel Goldschmidt: The metabolism of an isomer of xanthine and some isomers of the methylxanthines..... 83

Winfred W. Braman: A study in drying urine for chemical analysis... 105

Martha Tracy and Elizabeth E. Clark: The excretion of creatinine by normal women..... 115

Frank P. Underhill and Norman R. Blatherwick: Studies in carbohydrate metabolism. VII. The influence of subcutaneous injections of dextrose and of calcium lactate upon the blood sugar content and upon tetany after thyreoparathyroidectomy. 119

E. B. Hart and G. C. Humphrey (with the coöperation of J. J. Willaman and A. R. Lamb): The comparative efficiency for milk production of the nitrogen of alfalfa hay and the corn grain. (Preliminary observations on the effect of diuresis on milk secretion)..... 127

Donald D. Van Slyke and Glenn E. Cullen: The mode of action of urease and of enzymes in general..... 141

Donald D. Van Slyke and Gotthard Zacharias: The effect of hydrogen ion concentration and of inhibiting substances on urease. Further study on the mode of enzyme action..... 181

DONALD D. VAN SLYKE and GLENN E. CULLEN: A permanent preparation of urease and its use in the determination of urea..... 211
S. A. MATTHEWS and C. FERDINAND NELSON: Metabolic changes in muscular tissue. I. The fate of amino-acid mixtures..... 229
ARTHUR W. DOX and RAY E. NEIDIG: The soluble polysaccharides of lower fungi. II. Mycogalactan, a new polysaccharide in *Aspergillus niger*..... 235
WALTER W. PALMER, JAMES H. MEANS and JAMES L. GAMBLE: Basal metabolism and creatinine elimination..... 239
E. V. MCCOLLUM and MARGUERITE DAVIS: Observations on the isolation of the substance in butter fat which exerts a stimulating influence on growth..... 245
E. C. KENDALL: The determination of iodine in connection with studies in thyroid activity..... 251
HOWARD S. REED: The formation of hexone and purine bases in the autolysis of *Glomerella*..... 257
RAYMOND PEARL and FRANK M. SURFACE: Studies on the physiology of reproduction in the domestic fowl. IX. On the effect of corpus luteum substance upon ovulation in the fowl..... 263
GEORGE T. CALDWELL and H. GIDEON WELLS: The purine enzymes of the opossum (*Didelphis virginiana*)..... 279
PHILIP A. SHAFFER: On the determination of sugar in blood..... 285
PHILIP A. SHAFFER: On the normal level of blood sugar of the dog.... 297
ALBERT C. CRAWFORD and WALTER K. WATANABE: Parahydroxyphenylethylamine, a pressor compound in an American mistletoe..... 303
MIGUEL VERA and LEO LOEB: Immunization against the anticoagulating effect of hirudin..... 305
E. V. MCCOLLUM: The value of the proteins of the cereal grains and of milk for growth in the pig, and the influence of the plane of protein intake on growth..... 323
W. J. V. OSTERHOUT: The effect of alkali on permeability..... 335
OLAF BERGEIM, MARTIN E. REHFUSS and PHILIP B. HAWK: Gastrointestinal studies III (studies on water drinking XXI). Direct demonstration of the stimulatory power of water in the human stomach..... 345
E. B. HART and E. V. MCCOLLUM: Influence on growth of rations restricted to the corn or wheat grain..... 373
ALFRED W. BOSWORTH: The action of rennin on casein..... 397
H. STEENBOCK, V. E. NELSON and E. B. HART: Acidosis in omnivora and herbivora and its relation to protein storage..... 399
MAX MORSE: The effective principle in thyroid accelerating the involution in frog larvae..... 421
JACQUES LOEB: Is the antagonistic action of salts due to oppositely charged ions?..... 431

W. G. Lyle, L. J. Curtman and J. T. W. Marshall: The catalytic reactions of blood. I. A study of some of the factors involved in the benzidine test for occult blood..... 445
F. M. Scales: The enzymes of *Aspergillus terricola*..... 459
G. W. Raiziss, H. Dubin and A. I. Ringer: Studies in endogenous uric acid metabolism..... 473
A. I. Ringer and G. W. Raiziss: The excretion of creatinine by human individuals on a prolonged creatine-free diet..... 487
W. J. V. Osterhout: The effect of acid on permeability..... 493
Carl L. Alsberg and William Mansfield Clark: The solubility of oxygen in the serum of *Limulus polyphemus* L. and in solutions of pure *Limulus* haemocyanin..... 503
P. W. Bridgman: The coagulation of albumen by pressure..... 511
Frank P. Underhill: The distribution of arsenic in a human body.. 513
W. J. V. Osterhout: Antagonism between acids and salts..... 517
Frank C. Gephart and Frank A. Csonka: On the estimation of fat in feces..... 521
Index to Volume XIX..... 533

STUDIES ON BLOOD FAT.

I. VARIATIONS IN THE FAT CONTENT OF THE BLOOD UNDER APPROXIMATELY NORMAL CONDITIONS.

By W. R. BLOOR.

(From the Laboratories of Biological Chemistry of Washington University, St. Louis, Mo.)

(Received for publication June 29, 1914.)

It has long been known that the fat content of the blood may vary greatly at times. For example during fat absorption the blood serum becomes milky with fat and lipaemia is frequently present in severe diabetes. But, while much time during the last few years has been devoted to the variations in the other constituents of the blood, it is only very recently that attention has been directed to the probable importance of variations in the character and amount of the fatty constituents.

The work of Terroine[1] indicates the significance of this line of investigation. He found that not only was the total blood fat (fatty acids and cholesterin) remarkably constant for the normal individual and to a lesser extent for the species, but the relation between the fatty acids and cholesterin (lipaemic constant) was also very constant for the individual under normal conditions. He believed that the lipaemic constant was of the same order as the glycaemic constant and like it, was probably the expression of an efficient regulation. The same idea had been previously mentioned by Freudenberg[2] who believed that the seat of the regulation was the liver. Mayer and Schaeffer[3] found that the relation between fatty acids and lecithin in the blood was remarkably constant for most animals.

The reason for the lack of interest in the blood fat has in all probability been partly the failure to appreciate its importance,

[1] Terroine: *Journ. de physiol. et de path. gen.*, xvi, p. 212.

[2] Freudenberg: *Biochem. Zeitschr.*, xlv, p. 467.

[3] Mayer and Schaeffer: *Journ. de physiol. et de path. gen.*, xv, p. 984.

but to a considerable extent also to the lack of suitable methods. The Kumagawa-Suto method has been used for this purpose by Terroine, but aside from the possible destruction of some of the more sensitive fatty substances by the severe treatment, this otherwise excellent method is undesirable because it requires a relatively large amount of blood (ordinarily 25 cc.), the drawing of which at frequent intervals might well be supposed to upset the normal mechanisms. A method which has been used to some extent clinically and which requires only a small volume of blood is the ultramicroscopic method first used by Neumann.[4] No claim for exactness is, however, made for this method.

The results reported below which are in the nature of exploratory investigations in this field were obtained by the use of the nephelometric method[5]—a method in which a good degree of accuracy is attained by a relatively gentle treatment and which requires only a small amount (2–3 cc.) of blood for a determination. The same sample may be used for a cholesterin determination and it is hoped also for lecithin.

Since it was desired first to know the variations in the fat content of the blood under approximately normal conditions, the experimental procedures used in this work were simple. They consisted in (1) feeding fat; (2) intravenous injection of various fat preparations; (3) fasting for a short period (five to seven days); (4) narcosis. The results obtained by the nephelometric method represent total fat (fatty acids + cholesterin) and no attempt has been made in this part of the work to determine the fat partition, although the above mentioned results of Mayer and Schaeffer, Weill, Terroine, etc., have shown that this side of the problem is probably of great importance.

The literature on blood fat is considerable, but as most of it has to do with pathological conditions, only the more significant contributions bearing on the subjects to be discussed will be mentioned, and those in the different divisions of the paper. For convenience only the results and essential details of the experiments will be given in the discussion, the experiments in full detail being given at the end of the paper.

[4] Neumann: *Wien. klin. Wochenschr.*, 1907, p. 851.

[5] Bloor: this *Journal*, xvii, p. 384, 1914.

Blood changes after feeding fat—Alimentary lipaemia.

It has been known since the time when blood-letting was a common practice that the blood serum becomes milky after fat feeding, but the quantitative aspect of fat changes in the blood due to fat absorption was first studied by Neumann.[6] He counted the fat particles (haemakonien) by means of the ultramicroscope and found after feeding butter (with bread) that either the maximum or a very high degree of increase in the fat particles was reached in about two hours. Neisser and Braüning[7] by the same method found that the fat particles began to appear in the blood serum in from one to two hours, reached the height of their concentration in six hours, and then began to diminish. The intensity of the changes varied with the kind of fat fed and with the animal. It may be questioned whether the ultramicroscopic appearance really represents the true condition of the fat of the blood since Reicher[8] found by this method that in chloroform narcosis the particles in a given field increased from 2 to 95, while the increase by chemical measurement was only from 0.5 per cent to 1.6 per cent. Lattes[9] using the Kumagawa-Suto method found that after feeding fat there was an increase in the blood fat up to double the normal value.

Experiments on fat feeding. Experiments on the effect of feeding fat were carried out on normal animals and in one case after ligating the thoracic duct. After a preliminary fasting period of twenty-four hours the animals were fed and samples of blood taken from the jugular vein at convenient times afterwards. The results are given in the chart below:

The results indicate that although the degree of alimentary lipaemia may vary somewhat in different animals and in the same animal at different times, probably as a result of different nutritional conditions, in general the curve of variation in blood fat in fat feeding appears to follow that of normal fat absorption, as given by Munk from observations on the chyle, *i.e.*, slight

[6] Neumann: *loc. cit.*

[7] Neisser and Braüning: *Zeitschr. f. exp. Path. u. Therap.*, iv, p. 747, 1907.

[8] Reicher: *Zeitschr. f. klin. Med.*, lxv, p. 235, 1908.

[9] Lattes: *Arch. f. exp. Path. u. Pharm.*, lxvi, p. 132.

changes during the first two hours, an increase to a maximum at about the sixth hour, then a decrease. Tying off the thoracic duct prevented any increase in blood fat, which does not neces-

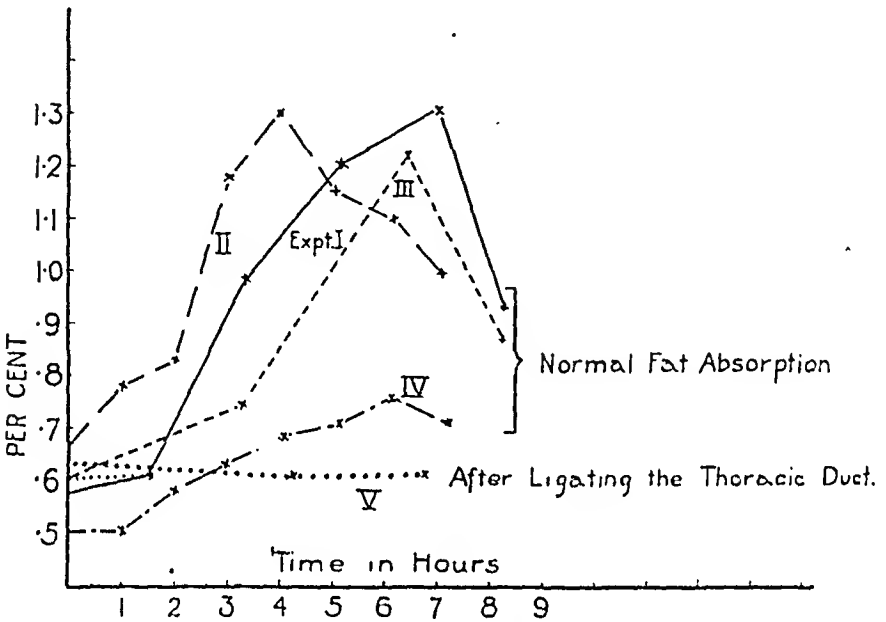


Diagram I. Changes in the fat content of the blood during normal fat absorption.

sarily mean that no absorption was taking place, but only that if there was any fat entering the blood stream it was being removed as rapidly as it entered.

Intravenous injections of fat.

Closely connected with the question of fat absorption from the intestine is that of the absorption of the fat from the blood by the tissues. Where and how quickly does the absorbed fat disappear from the blood? What is the mechanism of alimentary lipaemia? The injection of various fat preparation into the blood stream offered a way of studying these questions.

Injection of oils as such, is well known to cause embolism and death. If, however, the particles can be made small enough by emulsification or other form of suspension the injections

produce no apparent disturbance. Bondi and Neumann[10] injected colloidal suspensions of oils and found that they were well borne. Examination of the blood by the ultramicroscopic method showed that the injected fat disappeared from the blood in the course of a half hour. Injections of colored fat and iodized fat accumulated in the liver, bone marrow, spleen and muscles in about the order named.

Schott[11] in the course of experiments on intravenous feeding, injected egg yolk, which consists of about one-third of its weight of fat in very finely emulsified form, and found that the injections produced no more effect on the animal than the injection of an equal volume of salt solution. He found by ultramicroscopic examination that the fat particles disappeared from the blood in less than half an hour after injection.

Raper[12] injected casein emulsions of fats and found that much of the injected fat appeared unchanged in the liver. He made no examination of the fat of the blood.

Experiments on intravenous injections of fats. In the experiments recorded below fat suspensions of three kinds were injected intravenously: (1) casein emulsions of olive oil and cocoanut oil; (2) egg yolk; (3) colloidal suspensions of olive oil and cocoanut oil in water. Dogs were used throughout. The animals were fasted twenty-four hours before the injections. The injections were made into the jugular vein on the left side at the rate of 10–20 cc. per minute and blood samples were taken from the jugular vein on the right side.

Casein emulsions.

Casein emulsions were made with olive oil and cocoanut oil according to the directions of Raper (see above). The particles varied in size from 2 to 5μ (red blood cells about 7μ) and most of them showed Brownian movement.

Experiment I. 4.5 grams of fat injected. Estimated volume of blood 1000 cc. (one-fifteenth of body weight). Expected rise in blood fat = 0.45 per cent. Observed increase, none (see diagram II).

[10] Bondi and Neumann: *Wien. klin. Wochenschr.*, xxiii, p. 734, 1910.
[11] Schott: *Deutsch. Arch. f. klin. Med.*, cxii, p. 403, 1913.
[12] Raper: this *Journal*, xiv, p. 117, 1913.

EXPERIMENT II. Same animal. 4 grams of fat injected. No rise in blood fat.

EXPERIMENT III. Same animal. 3.75 grams of fat injected. No increase in blood fat.

In none of the experiments was there observed any increase in the quantity of the blood fat, although enough was injected in the experiments to raise the blood fat 50 to 90 per cent.

Egg-yolk. Thinking that perhaps the fault may have been in the size of the particles of the casein emulsions, which caused them to be strained out by the capillaries, some experiments were carried out with egg-yolk which is an excellent natural emulsion containing a high per cent of fat (fat, about 20 per cent; lecithin, 10 per cent) in a very fine state of division. For the injections the yolks of fresh eggs (one day old) were freed from white by washing with sterile salt solution, diluted with an equal volume of the salt solution, filtered first through absorbent cotton, then through filter paper and warmed to body temperature. The particles were almost entirely 1 to 2μ diameter and showed violent Brownian movement.

EXPERIMENT I. Fat injected, 10 grams. Estimated volume of blood, 1100 cc. Expected increase, 0.9 per cent (from 0.6 to 1.5 per cent); observed increase, 0.8 per cent (0.6 to 1.4 per cent). The observed increase accounted for 8.8 grams of the 10 grams of fat injected. The blood fat increased rapidly to a maximum eighteen minutes after the injection was completed, then decreased, at first rapidly then slowly until the normal fat value was reached in about seven hours (see diagram II).

EXPERIMENT II. In order to determine whether the nutritional condition of the animal had any effect on the rate of disappearance from the blood of the injected fat, a similar experiment was performed on the same dog after it had been fasted for eight days. Amount of fat injected, 12.8 grams. Fat accounted for in the blood as above, 9.9 grams. Expected increase in blood fat, 1.1 per cent (0.6 to 1.7 per cent). Observed increase, 0.9 per cent (0.6 to 1.5 per cent). Five minutes after the injection, the blood fat had reached 1.5 per cent (normal, 0.6 per cent), it then decreased rapidly (to 0.8 per cent in twenty-five minutes), then more slowly with a second rise (due probably to reabsorption) about the fourth hour after injection. In the fasting animal the disappearance of the injected fat was much more rapid than in the same animal in normal nutrition. These results are shown in diagram II.

Colloidal suspension of fat. In view of the current belief that the absorbed fat enters the blood stream with but little admixture

of other substances (except the lymph), it seemed of interest to know what happens on the injection of a fine suspension of pure fat in salt solution alone. To make these suspensions the fats were dissolved in hot alcohol and run with stirring into distilled water. The solution was filtered through a moist paper, evaporated to get rid of the alcohol and concentrated till it contained about 3 per cent of fat. It was then centrifugated to get rid of the larger particles, the lower layers drawn off and sufficient strong salt solution added to make the salt concentration about 0.7 per cent. The suspensions so prepared did not separate in the course of two weeks and contained nothing but fat, salt and water. The particles were about 1μ in diameter and were in violent Brownian movement. The preparation examined under the microscope resembled very closely chyle from the thoracic duct. Two injection experiments were carried out on the same animal, in one of which 0.5 gram of cocoanut oil and in the other 2.2 grams of egg fat were used. No change in the quantity of the blood fat could be detected in either case, although in the second experiment enough fat was injected to raise the percentage of fat in the blood from 0.60 to 1.15 per cent.

The results of all the fat injection experiments are expressed on the diagram on the following page.

No appreciable change in the blood fat was observed as the result of injections into the blood stream of either casein emulsions or colloidal suspensions of fat, while a very marked increase with, however, a somewhat rapid fall was obtained on injecting egg yolk. Several possibilities suggest themselves in explanation of these differences in the behavior of the fat. The rapid disappearance of injected fat suggests the presence of a storehouse, possibly the liver, where a certain quantity of fat may be quickly laid away, and which is quite distinct from the permanent fat depôts where storage takes place more slowly and which is responsible for the slow disappearance of the remainder of the fat. Since the quantity of fat injected in the case of egg-yolk (10–13 grams = 0.6–0.8 gram per kilo) was greater both relatively and absolutely than in the other injections (casein emulsion, 3.5–4.5 grams = 0.2–0.3 gram per kilo; colloidal suspensions, 0.5–2.2 grams = 0.1–0.4 gram per kilo) the increase of fat in the blood may have been due to the introduction of a quantity of fat greater than the temporary storehouse

could take care of, with a resulting accumulation. The fact of the more rapid disappearance of a larger amount of fat in the fasting animal (Exp. II, p. 6) bears out this idea, since in a fasting animal the temporary storehouse would presumably be empty.

Again, the egg-yolk fat consists of about one-third its weight of lecithin and it has been shown by Nerking[13] that injected lecithin persists for a long time in the blood. The amount of lecithin present in the egg-yolk fat, while not of itself sufficient to sustain

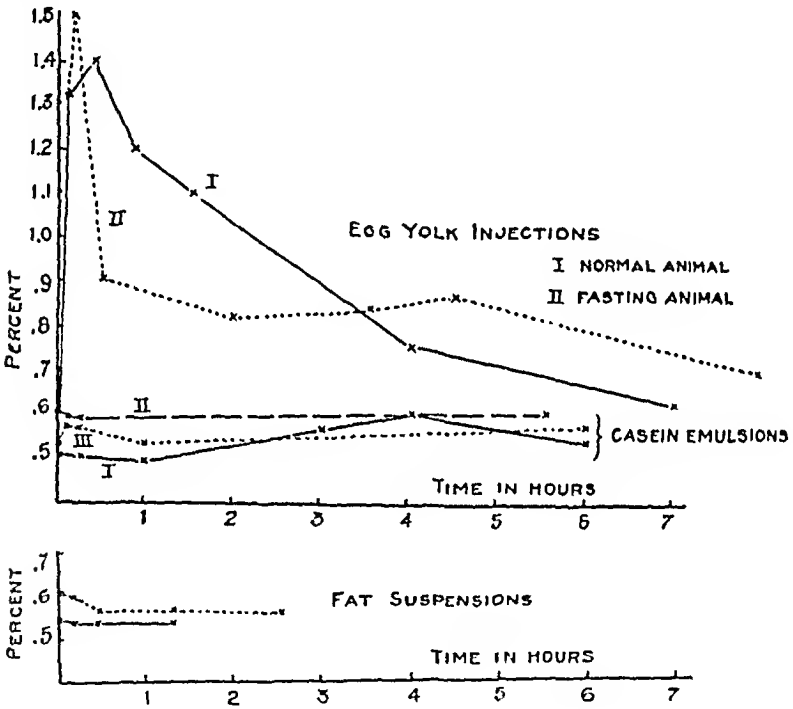


Diagram II. Intravenous injections of various fat preparations.

the level of blood fat, might have done so by slowing the removal of some of the other fats.

The bearing of these results on alimentary lipaemia—the accumulation of fat in the blood during fat absorption which may reach two or three times the normal fasting value—is not quite clear. The experiments show that preparations of pure triglycerides when injected in small quantities, disappear quickly from the

[13] Nerking: *Munch. med. Wochenschr.*, 1909, p. 1475.

blood. How then can we explain the persistence in the blood during fat absorption of what is ordinarily assumed to be practically pure triglycerides? In this connection the remarkable observation of Reicher[14] is significant. He found that after feeding pure triglycerides the accumulation of fatty substances in the blood consisted not only of triglycerides, but also of large proportions of lecithin and cholesterin. If this observation is correct, the persistence of the fat in the blood during fat absorption might be explained on the assumption mentioned above, that the lecithin slows the removal of the fat from the blood. The assumption of a temporary fat storehouse of limited capacity would also explain the phenomenon. A small amount of fat could be accommodated at once, while amounts above the capacity of the temporary storehouse would accumulate and be removed only as rapidly as the permanent fat depôts could take care of them.

Fasting.

After the first two or three days, the fasting organism depends mainly for its support on its fat stores. The increased use of fat would tend to increase the blood fat because of the greater amount transported, and the cellular break-down would also tend to increase it, because of the liberation of the fatty constituents. On the other hand, the increased call for fat by the energy-producing part of the organism would tend to decrease it owing to more rapid removal. It is conceivable that the resultant change in the blood fat of these opposing influences might depend on the one hand on the readiness with which the fat in the fat stores was available, and on the other, on the rapidity with which fat was being burned. It might be expected then that the blood fat at times would be increased during starvation and at other times not.

Schulz[15] (using the Dormeyer digestion method of fat-determination), found that the blood of fasting animals contained 50 to 100 per cent more fat than that of fed ones. Daddi[16] fasted animals for long periods and found (also by the use of the Dormeyer method) that there was, with two exceptions, an increase in blood fat in the first week, then a fall and about two weeks before death,

[14] Reicher: *Verhandl. d. Cong. f. inn. Med.*, xxviii, p. 327, 1911.
[15] Schulz: *Pflüger's Archiv*, lxv, p. 299.
[16] Daddi: *Arch. ital. de biol.*, xxx, pp. 437 and 439, 1898.

a further fall. Lattes,[17] using the Kumagawa-Suto method, found that the increase in starvation was slight if any, and hinted that the work of Schulz and Daddi would bear repetition.

Freudenberg[18] (using the Kumagawa-Suto method) found that there was no marked increase in the blood fat of fasting dogs. In a fat dog he found a 14 per cent increase on the fourth day and in the lean dog a 2 per cent increase in eleven days. As it was found by Daddi that the blood fat is lower in the second week of the fast than during the first week, this might account for the low value obtained in Freudenberg's second experiment.

These results vary widely. The differences may be partly due to the different methods of fat determination, but quite apart

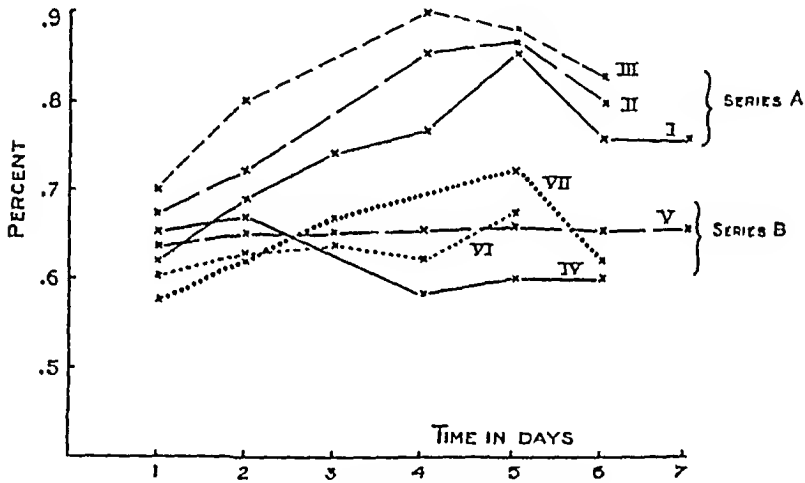


Diagram III. The effect of fasting on the fat content of the blood.

from the methods it is not unreasonable to expect that they might also be explained by differences in the conditions mentioned above—notably the relative availability of the stored fat and especially the fat in the temporary storehouses on the one hand and the rate of utilization on the other. The experiments reported below illustrate this possibility.

Experiments on the effect of fasting on the fat content of the blood. Dogs chosen at random were fasted for from five to seven days and daily determinations of the blood fat were made. The results are shown on the diagram above.

[17] Lattes: *loc. cit.*

[18] Freudenberg: *Biochem. Zeitschr.*, xlv, p. 467.

As may be seen from the diagram, the dogs fell into two classes as regards the behavior of the blood fat during the fasts. In dogs 10, 11 and 12, Series A, Experiments I, II, and III on diagram III there was an increase in the blood fat in fasting up to about the fifth day and after that a decrease. In dogs 15, 18 and 19, Series B, Experiments IV, V, and VI on the diagram there was no increase and in 15 a slight decrease. Although no attempt has been made to choose the dogs according to their degree of fatness or leanness and although there was no visible difference in the nutritional condition of the animals, it was decided to test whether the nutritional condition of the animal had any influence on the blood fat changes in starvation. Dog No. 18, Experiment V, which had shown no change in blood fat during a week's fasting was stuffed with fat meat, milk, olive oil, etc., for a week and then again fasted. The results of the second fasting are shown in the curve VII. As may be seen, the curve was remarkably similar to the fasting curves obtained in Experiments I, II, and III. There was the same increase of blood fat during the first five days and a similar falling off at the end of that time. The result then appears to indicate that the increase in blood fat in starvation depends on the nutritional condition of the animal. The findings are in agreement with those of Rosenfeld who could not produce fatty infiltration by phosphorus poisoning in lean dogs.

Narcotics.

Bibra and Harless,[19] the year after the discovery of the anesthetic action of ether by Morton and Jackson, in seeking a reason for the anesthesia, found that the ether-soluble material of the brain was decreased after repeated ether anesthesia, while that of the liver was increased. They believed therefore that anesthesia was produced by dissolving out the brain lipoids. This plausible hypothesis held the field until the investigations of Meyer and Overton on the relative solubility of narcotics in water and in fatty substances gave support to the theory that narcosis is produced by disturbances of the normal mechanisms of the cell induced by the presence of the narcotic which has been selectively absorbed from the blood.

[19] Bibra and Harless: Quoted from Reicher, *Zeitschr. f. klin. Med.*, lxv, p. 235.

Reicher[20] found that the blood fat might be increased up to 300 per cent of the normal by various narcotics and that the increase was due about equally to fat, cholesterin and lecithin. His findings might be regarded as supporting the old Bibra and Harless theory of narcosis, although Reicher was more inclined to the belief that the increase of lipoids was in the nature of a protective mechanism—the lipoids in the blood dissolving the narcotic and thus protecting the cells.

Nerking,[21] on the basis of Reicher's protection hypothesis, carried out some experiments in which he found that the injection of lecithin into the blood stream did have a marked effect on the narcosis. More of the narcotic was needed to produce narcosis and the animals recovered more quickly and with less after effects than without the injection.

Kramer[22] in repeating the work, criticised Nerking's experiments because his doses of anesthetic were not properly measured. With accurately measured doses of the anesthetic (ether intravenously in salt solution) he could not obtain these results.

The findings of Reicher regarding the increase in blood fat during narcosis are disputed by Lattes,[23] who could find no increase in the blood fat in chloroform narcosis even though the time of anesthesia was double that of Reicher.

The presence in the cells of a substance active enough to produce narcosis would be liable to bring about more or less injury to the cells with resulting breakdown and setting free some of the protoplasmic constituents, among them the fatty substances. Necrosis of the tissues, especially the liver, as a result of chloroform anesthesia has been noted many times, and Howland and Richards[24] state that even a short anesthesia with chloroform was sufficient to produce a necrosis which differed only in degree from that produced by long narcosis. The products of the necrosis would tend to accumulate in the blood and if they did so, would explain the "after rise" in blood fat (noted below) during the days following a narcosis.

[20] Reicher: *Zeitschr. f. klin. Med.*, lxv, p. 235.
[21] Nerking: *loc. cit.*
[22] Kramer: *Journ. Exp. Med.*, xvii, p. 206.
[23] Lattes: *loc. cit.*
[24] Howland and Richards: *Journ. Exp. Med.*, xi, p. 344, 1909.

Experiments on the effects of narcotics on the fat content of the blood. Experiments were carried out with ether, alcohol, morphine and chloroform. Dogs were used and there was in all cases a fast of twenty-four hours before the experiment. Ether and chloroform were given by inhalation during three-hour periods, the anesthesia being carried just to the abolition of the corneal reflex. Alcohol was given by stomach tube in 15 per cent water solution. Morphine was given subcutaneously in 4 per cent solution. Quanti-

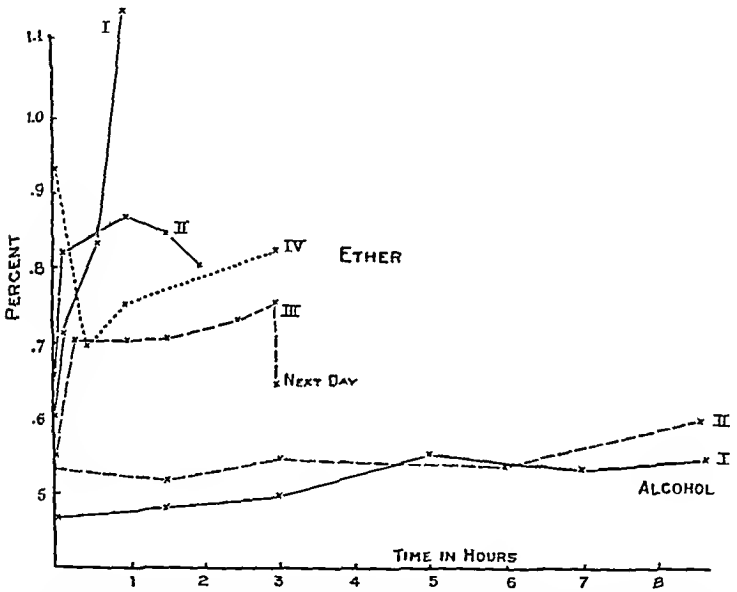


Diagram IV. The effect of narcotics on the fat content of the blood—ether and alcohol.

ties of both the latter were given until the animals lay helpless on the floor, but were not entirely unconscious. The results of the experiments are condensed in the diagrams below. The details are given at the end of the paper.

Ether. In all the ether treated animals Experiments I, II, and III, diagram IV, there was a decided rise in the blood fat during the anesthesia. In Experiment I in which the dog died after one hour, the rise was continuous. In Experiments II and III where

the animals recovered, there was a sharp rise at first followed by a slower rise or by a decrease. Experiment IV is interesting because the dog had inadvertently been fed before the anesthesia and fat absorption was in progress as evidenced by the high fat content of the blood. The effect of the ether was first to stop the fat absorption and second to produce what was probably the regular rise observed in the other cases.

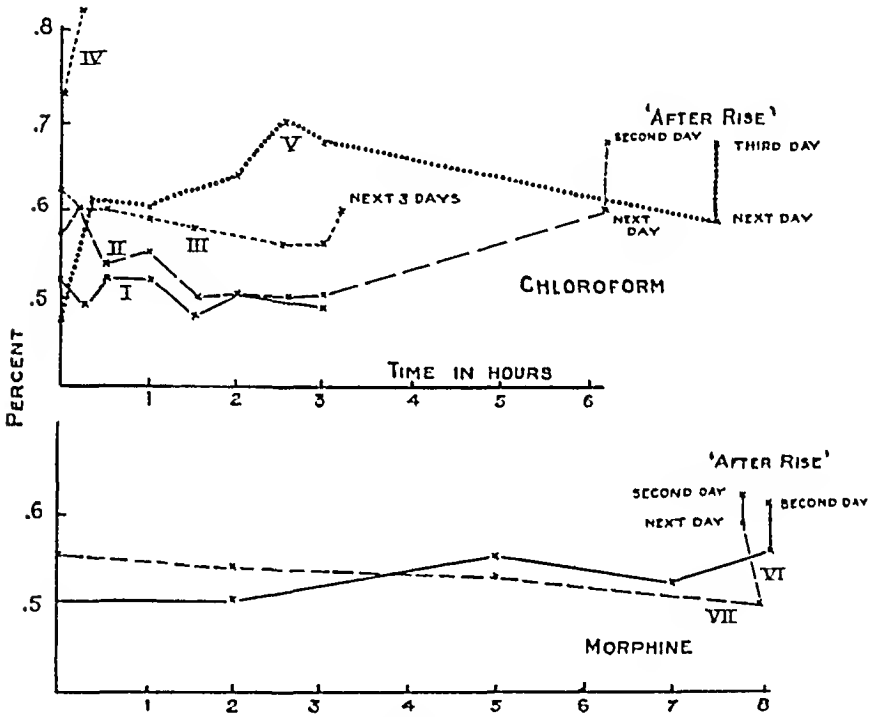


Diagram V. Effect of narcotics on the fat content of the blood. Chloroform and morphine.

Alcohol. The effect produced by alcohol was small. There was a slight increase in blood fat in the course of eight or nine hours.

Chloroform. In Experiments I, II, III, diagram V, there was no increase in blood fat during the narcosis, but in all cases a slight fall. The dog in Experiment II recovered very slowly from the anesthetic and remained dull for two or three days and it was therefore thought of interest to examine the fat in his blood

during this time. There was found a decided increase (see diagram V). This "after rise" in the blood fat will be referred to later.

In order to test the possibility that, as in fasting (which see, diagram III, Experiments V and VII), the fatty condition of the animal may have an influence on the changes in the blood fat, comparative experiments were carried out with dog 18. After a fast of eight days, she was anesthetized with chloroform for three hours and the blood fat examined. As may be seen from the diagram (Experiment III), there was no rise in the blood fat either during the anesthesia or in the course of the next three days. The animal was then stuffed with fat for a week and again anesthetized. Death ensued within ten minutes. Now while death under chloroform is not an unusual occurrence, I am inclined to attach some significance to it in this case in view of the fact that the same animal had safely stood a three hours' anesthesia a short time before and also because the blood fat in this animal had increased from 0.73 to 0.81 per cent in the course of the few minutes of anesthesia (Experiment IV, diagram V). Dog 10, which died under ether, showed a similar rapid rise (see diagram IV, Experiment I).

A similar experiment carried out with dog 20 resulted in a marked rise in the blood fat during the anesthesia and also a considerable after rise (Experiment V, diagram V).

Morphine. There was no appreciable rise during the first eight hours of the narcosis, but a considerable after rise in both in the course of the next two days (Experiments VI and VII, diagram V).

Summary of the results of narcosis. 1. During the narcosis. The effects on the fat content of the blood produced by the narcotics as far as observed were as follows: (*a*) Ether always produced a definite rise in blood fat of from 40 to 100 per cent during the anesthesia, the rate of rise being most marked during the first hour. (*b*) Morphine and alcohol produced slight or no increase. (*c*) Chloroform had no effect on the fat content of the blood (up to three hours) unless the animal had been stuffed with fat food for some time previously, when there was a marked rise (40 per cent). In the two cases where death occurred during the narcosis (dog 10, Experiment I, diagram IV with ether, and dog 18 with chloroform, Experiment IV, diagram V) there was a sharp rise in the blood fat which continued until the death of the animal.

2. After effects. With chloroform and morphine there was in most cases an "after rise" of from 10 to 40 per cent during the two or three days after the narcosis. No "after rise" was observed with ether (one experiment).

Variations in the fat content of the blood of dogs under normal conditions.

In the course of the experiments reported above, determinations of the blood fat of a considerable number of dogs had been made over varying periods of time up to two months. Since many of the determinations were made while the animals were in a normal state of health and nutrition, it seemed desirable to collect the values so obtained so as to obtain an idea of the variations in the fat content of the blood under normal conditions. All determinations were made on samples of blood taken twenty-four hours after the last feeding. The percentage results are collected in the table below.

DOG NO.	FAT CONTENT OF THE BLOOD AT VARIOUS TIMES	AVERAGE	VARIATIONS FROM THE AVERAGE
10	0 58; 0.61	0.59	2.0
13	0 67; 0.65	0.66	1.6
14	0 60; 0.60	0.60	0.0
15	0 60; 0 67	0.64	6.0
16	0 52; 0.53: 0.55; 0 61	0.55	10.0
17	0 51; 0.50; 0 60; 0 52; 0.58; 0.47; 0.49; 0 58	0.53	12.0
18	0.60; 0 64; 0 65; 0 60	0 62	3.3
19	0.55; 0 51; 0.54; 0 62; 0.60	0.57	8.0
20	0 50; 0 48; 0.56	0.51	10.0
Average for all dogs.		0.59	

Greatest variation of each dog from his average..... 12 per cent
Average variation of each dog from his average..... 6 per cent
Greatest variation of any animal from the average for all. ..... 14 per cent
Average variation of any animal from the average for all. ..... 7 per cent

The blood fat of individual dogs thus appears to be quite constant and that of different dogs is almost as constant.

Terroine[25] found variations of as high as 113 per cent between maximum and minimum figures for different individuals and 30 per cent in the same animals. These were extreme figures, the average was much lower and the variations from the average as calculated above were still lower, so that his results are in good agreement with the above, although obtained by a different method. Terroine's average value for the fat content of dogs' blood was 0.45 per cent, while that found above by the nephelometric method was 0.59 per cent, a difference which is probably explainable by the difference in methods, the nephelometric method giving higher values than the Kumagawa-Suto method.[26]

SUMMARY AND DISCUSSION.

Feeding fat in the ordinary way caused an increase in the fat content of the blood beginning in about one hour after feeding and reaching a maximum in about six hours. When the thoracic duct was tied there was no increase in blood fat after fat feeding.

Intravenously injected fat as casein emulsions or in colloidal suspension up to 0.4 gram per kilo of body weight—enough to increase the fat content 100 per cent—disappeared from the blood in less than five minutes. Since the particles were from one-half to one-seventh the size of the red blood corpuscles, the disappearance could scarcely have been due to a straining out by the capillaries, but rather to a storage in some way or other, possibly by the liver, since it has been shown that the injected fat accumulates in largest amount in the liver. When amounts of fat up to 0.8 gram per kilo in the form of egg yolk were injected, some of the fat persisted in the blood for several hours, which might have been due to the flooding of the temporary storehouses beyond their capacity, or to the influence of the lecithin in the egg yolk fat.

Fasting of from five to seven days may or may not produce an increase in the blood fat, depending apparently on the nutritional condition of the animal. An animal which did not ordinarily show any increase in blood fat in fasting, was made to exhibit the usual curve of increase of blood fat in fasting by stuffing with fat food for a week before the fast.

[25] Terroine: *loc. cit.*

[26] Bloor: this *Journal, loc. cit.*

Of the narcotics used, ether was the only one which ordinarily produced a rise in the fat content of the blood during the narcosis. The fact that ether is soluble in water to about 10 per cent, suggests that the increase may have been due to the increased solubility of the fatty substances of the tissues in the blood-ether mixture.

Chloroform produced a rise in blood fat only when the animal had been stuffed with fat for some time previously. The "after rise" in the blood fat, *i.e.*, an increased blood fat content during the two or three days following narcosis was observed after chloroform and morphine but not after ether (only one case examined). The after rise may be the expression of necrosis of the tissue cells with liberation of their fatty constituents.

The fat content of the blood of dogs is shown to be quite constant both for the individual and the species, and indicates that there is an efficient regulation. The constancy of fat values in the blood of dogs gives reason to expect a similar constancy in the normal blood fat values of human beings, and indicates that blood fat determinations would give valuable diagnostic information in certain conditions.

The results as a whole indicate that fat may be stored in two ways: (1) a temporary storage where the fat may be quickly but loosely stored and whose capacity is limited, and (2) the permanent fat depôts which take up fat slowly and release it slowly. The temporary storehouse may be a part of the fat-regulating mechanism, but at any rate the loosely stored fat may be quickly released by certain stimuli, as for example, chloroform anesthesia and the sudden flooding of the organism with fat may be a potential source of danger. In the animals that died under the anesthetic there was observed a rapid and continuous rise in the fat content of the blood during the anesthesia until death.

NOTE. Since the above was written, a paper has appeared by Terroine[27] on the effect of long fasts and of fat feeding on the content of the blood in fatty acids and cholesterin. In long fasts up to the death of the animal, he finds no constancy in the variations in the fatty acids. Sometimes they increase and sometimes decrease as the fast proceeds, due as he believes to differences in the fat reserves of the different animals. Cholesterin

[27] Terroine: *Journ. de physiol. et de path. gen.*, xvi, p. 386.

progressively diminishes in all animals. Feeding fat produces an increase in the fatty acids and cholesterin in the blood to a maximum about the sixth hour. The maximum increase varies from 12 to 76 per cent. The parallel increase in cholesterin and fatty acids is in agreement with the findings of Reicher mentioned above. Terroine's conclusions regarding the effect of fasting are in accord with those expressed in the above paper in that the changes in the fat content of the blood in fasting are believed to be dependent on the nutritional condition of the animal. The effects of feeding fat on the fat content of the blood are also in agreement with those recorded in this paper although quantitatively much less marked.

PROTOCOLS OF THE EXPERIMENTS.

Alimentary lipaemia.

I. Dog 10. Weight 5 K. Previously fasted 24 hours. Fed lean meat and 30 cc. olive oil at 9.20.

Blood fat before feeding, 0.58 per cent; 1½ hours after, 0.60; 3¼ hours after, 0.96; 5 hours, 1.18; 7 hours, 1.28; 8 hours, 0.92; 24 hours, 0.61; 26 hours, 0.62 per cent.

II. Dog 13. Experiment 1. Weight 13 K. Fasted 24 hours previously. Fed 100 grams meat and 30 cc. olive oil.

Before feeding blood fat, 0.6 per cent; 1 hour after, 0.79; 2 hours, 0.82; 3 hours, 1.16; 4 hours, 1.29; 5 hours, 1.14; 6 hours, 1.09; 7 hours, 0.98; 8 hours, 0.96 per cent.

III. Dog 14. Weight 11 K. Fasted 24 hours previously. Fed 125 grams lean meat and 100 cc. of olive oil.

First sample, just before feeding, 0.6 per cent; 3¼ hours after, 0.73; 6¼ hours after, 1.2; 8 hours after, 0.87 per cent.

IV. Dog 13. Experiment 2. Weight 13 K. Previously fasted 48 hours. Fed 50 grams meat and 50 cc. olive oil at 8.45.

First sample, just before feeding, blood fat content, 0.50 per cent; 1 hour later, 0.50; 2 hours later, 0.56; 3 hours after, 0.62; 4 hours, 0.68; 5 hours, 0.70; 6 hours, 0.75; 7 hours, 0.7 per cent.

V. Tying off the thoracic duct. Dog 13. The normal alimentary lipaemia in this dog with the same feeding (see Experiment IV, diagram I) was about 50 per cent increase over the normal. The duct was tied off the day before the feeding and the animal had apparently made a good recovery and had a good appetite. He was fed 50 cc. of olive oil and one roll at 9.15. First blood sample, just before feeding, fat content = 0.62 per cent; 4 hours later, 0.60; 6½ hours later, 0.60 per cent. There was then no increase in the blood fat. If absorption were taking place by other channels it was so slow that the fat was removed from the blood as rapidly as it entered it.

Next day the dog was sick, had no appetite and showed signs of severe intestinal disturbance—much diarrhoea with watery stools. The diarrhoea persisted for the next two days with considerable blood in the stools.

Intravenous injections of fat.

A. Casein emulsions. Experiment I. Dog 17. Weight 16 K. in good condition. 30 cc. of a 15 per cent emulsion of olive oil (4.5 grams) were injected into the left jugular vein, the injection lasting two minutes. Samples of blood were taken before injection and at intervals up to about 6 hours afterwards. The sample before the injection was taken from the left jugular vein, the others from the right.

Estimated volume of blood, 1000 cc. Fat injected, 4.5 grams. Expected increase in blood fat, 0.45 per cent. Increase found, none. Before injection blood fat, 0.50 per cent; 10 minutes after, 0.50; 1 hour after, 0.49; 3 hours after, 0.58; 4 hours after, 0.60; 6 hours after, 0.52 per cent. The injected fat disappeared at once from the blood stream.

Experiment II. Same animal. Injected 40 cc. of a 10 per cent emulsion (4 grams of fat) during 3 minutes. Before injection blood fat = 0.60 per cent. 3 minutes after injection, 0.58; 15 minutes after, 0.58; 5½ hours after, 0.60 per cent. The injected fat disappeared as before.

Experiment III. Same animal. Injected 50 cc. of a 7.5 per cent emulsion (3.75 grams of fat), in the course of 15 minutes. Before injection blood fat, 0.52 per cent; 3 minutes after, 0.58; 10 minutes after, 0.57; 1 hour after, 0.52; 6 hours after, 0.51 per cent.

In none of these experiments could there be found any definite increase in the blood fat injections of the casein emulsion, although enough was injected to raise the fat content about 50 per cent.

The symptoms on injection were the same in all cases—slight and transitory respiratory disturbance (increased rate and depth of respiration) during injection, slight vomiting of bile-stained fluid, diarrhoea with greenish watery stools. The experiments were made at intervals of about a week, during which time the animal was on an abundant mixed diet. Before the injections the animal was fasted for 24 hours.

B. Egg yolk injections. Thinking that perhaps the casein emulsion was too coarse, experiments were next carried out with egg-yolk which is a very fine natural emulsion containing about 30 per cent of fat (including 10 per cent lecithin). Schott[28] had used it for intravenous feeding and found that the fat had disappeared from the blood in about one hour (ultramicroscopic examination). For use in these experiments the yolks from three fresh eggs were freed from the white by washing with sterile salt solution, diluted with equal parts of the salt solution, filtered through absorbent cotton and filter paper, then warmed for injection. Examined microscopically, the preparation consisted of a suspension of very fine particles, about 1–2μ in size and with strong Brownian movement.

Experiment I. Dog No. 18. Female. Weight 17 K. In fair condition. Last feeding, 24 hours previously. Injected 62 cc. of the emulsion (16 per cent) containing about 10 grams of fat during 8 minutes. There

[28] Schott: *loc. cit.*

was a slight respiratory acceleration and towards the end of the injection a slowing of the heart rate, for which reason the injection was stopped. After leaving the table the animal showed no further abnormal symptoms.

Before injection, blood fat = 0.60 per cent; 3 minutes after (right jugular), 1.31 per cent; 18 minutes after, 1.4; 50 minutes after, 1.2; 1½ hours after, 1.1; 4 hours after, 0.77; 7 hours after, 0.60 per cent.

There was in this case an increase in the total fat content of the blood of about 0.8 per cent (130 per cent of normal). The blood volume of the animal (one-fifteenth of body weight) = 1100 cc. On this basis the excess of fat in the blood was 8.8 grams (amount injected = 10 grams) which accounts for practically all of the fat injected.

Experiment II. Same animal, but which had been fasted for 8 days before the experiment. 80 cc. of an egg yolk mixture containing 16 per cent of fat was injected during ten minutes. Blood fat before injection = 0.6 per cent; 5 minutes after, 1.5; 30 minutes after, 0.9; 2 hours after, 0.81; 3½ hours after, 0.82; 4½ hours after, 0.86; 8 hours after, 0.70 per cent.

Fat injected, 12.8 grams. Accounted for by fat increase in blood, 9.9 grams.

In this experiment the fat disappeared from the blood with greater rapidity than in the same dog in good nutritional condition.

C. Fat suspension injections. Experiment I. Dog 19, male, black and tan, weight 5 K. Injected 17 cc. of a 3 per cent water suspension of cocoanut oil (0.5 gram of fat) during 3 minutes. Blood fat before injection = 0.55 per cent; 5 minutes after completion of injection, 0.53; 25 minutes after, 0.53; 1¼ hours after, 0.52 per cent.

Approximate volume of blood = 350 cc. Enough fat had been injected to raise the blood fat 25 per cent. No increase was noted.

Experiment II. Same animal. Injected 75 cc. of a 2.9 per cent suspension of egg-yolk fat (freed from lecithin) = 2.2 grams of fat during 15 minutes.

Blood fat before injection = 0.60 per cent; 5 minutes after injection, 0.60; 25 minutes after injection, 0.56; 1¼ hours after injection, 0.56; 2½ hours after injection, 0.55 per cent.

Approximate blood volume, 350 cc.

Enough fat had been injected to increase the blood fat 100 per cent. No increase was noted. The injected fat had disappeared at once.

Fasting.

I. Dog 10. Weight 5 K. Female. Last feeding meat and fat 24 hours previously. 1st day fasting the fat content of blood was 0.61 per cent; 2nd day, 0.68; 3rd day, 0.74; 4th day, 0.76; 5th day, 0.86; 6th day, 0.75; 7th day, 0.75 per cent.

II. Dog 12. Weight 11 K. Male. Last feeding bread and meat 30 hours previously. 1st day fasting the fat content of the blood was 0.67 per cent; 2nd day, 0.72; 4th day, 0.85; 5th day, 0.86; 6th day, 0.80 per cent.

III. Dog. 11. Weight 10 K. Male. Last feeding bread and meat 30 hours previously. Ist day fasting blood fat = 0.70 per cent; 2nd day, 0.80; 4th day, 0.90; 5th day, 0.87; 6th day, 0.83 per cent.

IV. Dog 15. Weight 5.5 K. Female, in fair condition. After 24 hours' fast the blood fat was 0.67 per cent; 2 days fasting, 0.69; 4 days, 0.58; 5 days, 0.60; 6 days, 0.60 per cent.

V. Dog 18. Weight 17 K. Female, rather thin. After 24 hours' fast blood fat = 0.64 per cent; 2 days, 0.65; 4 days, 0.64; 5 days, 0.66; 6 days, 0.65; 7 days, 0.65 per cent.

There was practically no change in fat content of the blood during fasting.

VI. Dog 19. Male, weight 5 K. Blood fat 30 hours after last feeding = 0.60 per cent; second day, 0.64; third day, 0.63; fourth day, 0.64; fifth day, 0.63; sixth day, 0.67 per cent.

VII. In order to test whether the nutritional condition of the animal had any influence on the blood fat changes, dog 18, Experiment V which had shown no increase in blood fat during a week of fasting, was stuffed with fat meat, milk, olive oil, etc., for a week and then again fasted.

Blood fat 24 hours after last meal = 0.58 per cent; second day, 0.62; third day, 0.66; fifth day, 0.72; sixth day, 0.62 per cent. After stuffing with fat this animal showed the same increase as those in the first three experiments.

Narcotics

A. Ether. Dog 10. Weight 5 K. Female. Fasted 5 days previously. Before the anesthetic the fat content of the blood was 0.60 per cent. 10 minutes after the beginning of the anesthetic, 0.71 per cent; 40 minutes after, 0.83 per cent; one hour after and after respiration had ceased, but before the heart had stopped beating, the blood fat had reached 1.15 per cent. The animal could not be revived. This dog was hard to anesthetize and took a great deal of ether.

II. Dog 13. Weight 13 K. Rather thin. Fasted 24 hours. Before anesthesia the fat content of the blood was 0.65 per cent; 10 minutes after beginning anesthetic, 0.82; 1 hour after, 0.86; 1½ hours after, 0.83; 2 hours after, 0.80 per cent. The animal bore the ether well.

III. Dog 20. Female. Weight 10 K. No food for 40 hours. Before that, well fed.

Blood fat before anesthetizing = 0.56 per cent; 15 minutes after beginning anesthesia, 0.7; 1 hour later, 0.70; 1½ hours later, 0.70; 2½ hours later, 0.73; 3 hours later, 0.75; next day, 0.62 per cent.

IV. Ether on fat absorption. Dog 14. Weight 11 K. Female. This dog had inadvertently been fed some fat meat a few hours before the experiment.

Sample 1 before the anesthetic = 0.94 per cent; 20 minutes after the anesthetic, 0.7; 50 minutes after, 0.75; 180 minutes after, 0.82 per cent. The ether stopped fat absorption and then later produced its regular effect of increasing the fat content of the blood.

B. Alcohol. Dog 17. Fat old male. Dog 16. Thin young female. Both dogs had been on mixed diet for one week. They were fasted 24 hours before the experiment. The alcohol, diluted to 15 per cent with water, was given by stomach tube.

Experiment I. Dog 17. Total dose, 50 cc. of 95 per cent alcohol. Blood fat before taking = 0.47 per cent. First dose, 25 cc. in 125 cc. of water 1½ hours later, 0.49 per cent. 10 cc. more alcohol given; 3 hours after first dose, blood fat = 0.50 per cent. Another dose of 15 cc. given; 5 hours after first dose, 0.56 per cent; 7 hours after first dose, 0.53 per cent; 8½ hours after first dose, 0.56 per cent.

There was a small but definite increase in the blood fat.

Experiment II. Dog 16. Total dose, 40 cc. of 95 per cent alcohol. First dose, 15 cc. in 75 cc. of water. Fat content of blood before anesthetic = 0.53 per cent; 1½ hours after, 0.52 per cent; gave a second dose of 10 cc. of alcohol; 3 hours after first dose blood fat = 0.55 per cent; gave a third dose of 15 cc. alcohol; 5 hours after first dose, 0.55 per cent; 6½ hours after, 0.54 per cent; 8 hours after, 0.59 per cent. A slight increase in the blood fat during the narcosis.

C. Chloroform. Experiment I. Dog 16. Thin young female. On fat-free diet for 10 days. Last meal night before. Readily anesthetized and required but little chloroform. Recovery was prompt. Next day to all appearances normal. Before anesthetic, blood fat = 0.52 per cent; just after the anesthesia was complete (abolition of corneal reflex), 0.49 per cent; 30 minutes later, 0.53; 1 hour after, 0.52; 1½ hours after, 0.47; 2 hours after, 0.50; 2¾ hours after, 0.48 per cent.

The results show no increase in blood fat during the anesthesia.

Experiment II. Dog 17. Large, rather fat old male on mixed diet. Last meal the night before.

Before anesthesia, 0.58 per cent; just after, 0.60; ½ hour after, 0.52; 1 hour after, 0.56; 1½ hours after, 0.50; 2 hours after, 0.50; 2½ hours after, 0.50; 3 hours after, 0.50 per cent.

Recovery from the anesthetic was slow and the animal was not normal for several days.

Blood fat next day after = 0.63 per cent; second day after, 0.67 per cent. There was then no increase in the blood fat during the anesthesia—rather a falling off. On the two days following the anesthetic, however, there was a definite increase in the blood fat the "after rise" which may be an expression of necrosis.

Since the significance of fat changes in the blood are entirely unknown, the following work was done with the idea of throwing some light on this subject and opening new channels of attack.

Experiment III. To determine whether the nutritional condition of the animal has any effect on blood fat changes during chloroform anesthesia, as was found to be the case in fasting. Dog 18, female, weight 15 K., was fasted for 8 days (there was a rise of blood fat during the fasting, see p. 22, Experiment VII) then anesthetized for three hours.

Fat content of the blood before anesthesia = 0.62 per cent; immediately after (3 minutes from beginning of anesthesia), 0.60; ½ hour after, 0.60; 1 hour after, 0.58; 1½ hours after, 0.56; 2½ hours after, 0.55; 3 hours after, 0.56. Anesthetic stopped. 5 hours later, 0.57; next morning, 0.60; second morning, 0.61; third morning, 0.61 per cent.

A slight fall in blood fat during the anesthesia with no after rise during the succeeding 3 days.

Experiment IV. The same animal was now given all the fat meat, milk, olive oil, etc., that she would eat for a week, fasted for 24 hours, and then anesthetized as before. Blood fat before anesthesia = 0.73 per cent. Died with respiratory failure 10 minutes after beginning the anesthetic. Blood fat at death, 0.81 per cent.

Experiment V. Dog 20. Female. Weight 10 K. was fed for 1 week on excess of fatty food, mostly fat meat, fasted 24 hours, then anesthetized for 3 hours.

Fat content of the blood before anesthesia = 0.48 per cent; 15 minutes after beginning the administration, 0.60 per cent; 1 hour after, 0.60; 2 hours after, 0.64; 2½ hours, 0.70; 3 hours, 0.69 per cent. Anesthetic stopped. 5 hours later, 0.59; next day, 0.62; third day, 0.66 per cent.

There was in this experiment a considerable rise during the course of the experiment and also a definite after rise.

D. Morphine. The same two dogs (16 and 17) as had been used for the alcohol and chloroform experiments. The morphine was given as a 4 per cent solution of the sulphate subcutaneously.

Experiment VI. Dog 17. Total dose, 11 cc. of 4 per cent solution. First dose, 7 cc. of 4 per cent solution.

Fat concentration in the blood before = 0.50 per cent; 2 hours after, 0.50; gave a second dose of 2 cc.; 5 hours after, 0.56 per cent; gave a third dose of 2 cc.; 7 hours after first dose, 0.52 per cent; 8½ hours after first dose, 0.56 per cent. Next day, 0.50 per cent. The second day, 0.60 per cent. This dog was dull and cross for two or three days.

Experiment VII. Dog 16. Total dose, 9 cc. of 4 per cent solution. First dose, 5 cc. of 4 per cent solution.

Blood fat before = 0.55 per cent; 2 hours later, 0.52; gave a second dose of 2 cc.; 5 hours after, 0.53; gave a third dose of 2 cc.; 8 hours after first dose, 0.50; next day, 0.59; second day, 0.61 per cent. This animal was active and apparently normal next day.

Morphine appears to produce no change in the fat concentration of the blood during the narcosis, but produces a considerable "after rise" during the two or three days following.

RESEARCHES ON PURINES. XV.[1]

ON 2-OXY-5-AMINO-6-ETHYLAMINOPYRIMIDINE AND 2, 8-DIOXY-9-ETHYLPURINE.

By CARL O. JOHNS and BYRON M. HENDRIX.

(From the Sheffield Laboratory of Yale University.)

(Received for publication, July 1, 1914.)

In a recent paper from this laboratory we described the method of preparation and the properties of 2,8-dioxy-6-methyl-9-ethylpurine (VII),[2] and in a following paper we discussed some 2-oxy-6-methyl-9-ethylpurine derivatives.[3] All of these compounds contained a methyl group in position 6. Since the properties of these ethyl purines would be modified considerably by the presence of the methyl group, it seemed of interest to prepare a simple purine derivative which contained only an ethyl group. We have therefore synthesized a number of new pyrimidines and these have furnished a means of obtaining 2,8-dioxy-9-ethylpurine (IV). The reactions involved in this work were as follows:

2-Ethylmercapto-6-chlorpyrimidine (I)[4] was heated with aqueous ethylamine and gave a quantitative yield of 2-ethylmercapto-6-ethylaminopyrimidine (II). This compound was an oil which did not solidify at 0°C. and which distilled under diminished pressure without undergoing decomposition. The corresponding unsubstituted pyrimidine, 2-ethylmercapto-6-aminopyrimidine (IX),[5] is a solid compound which cannot be distilled under diminished pressure. When 2-ethylmercapto-6-ethylaminopyrimidine was was boiled with hydrochloric acid, a hydrochloride of 2-oxy-6-

[1] Johns: this *Journal*, xvii, p. 1, 1914. The present investigation was aided by a grant from the Bache fund.

[2] Johns and Baumann: this *Journal*, xv, p. 119, 1913.

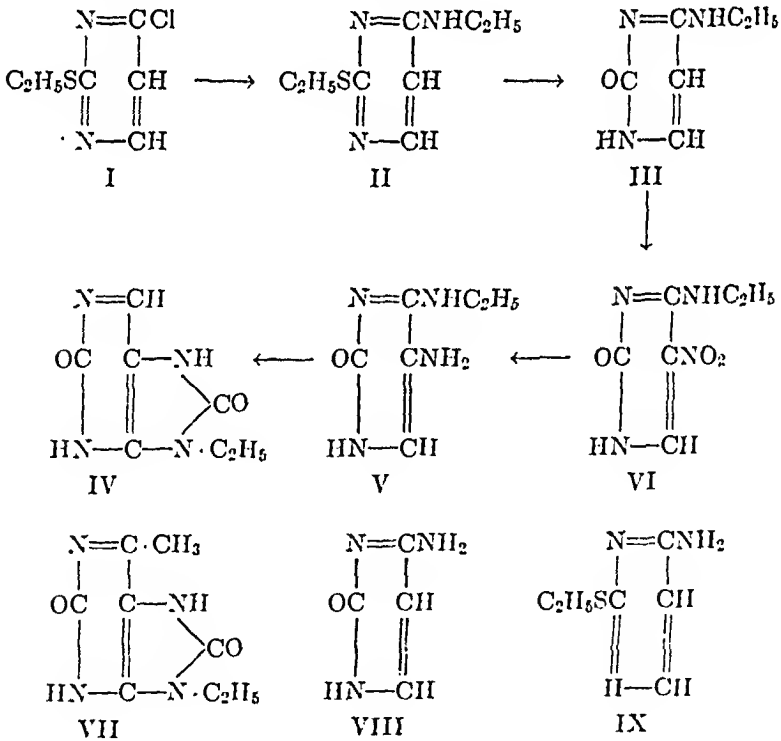
[3] Johns and Baumann: *ibid.*, xv, p. 515, 1913.

[4] Wheeler and Johnson: *Amer. Chem. Journ.*, xxix, p. 496, 1903.

[5] Wheeler and Johnson: *ibid.*, xxix, p. 497, 1903.

ethylaminopyrimidine was obtained (III). The free base was isolated by means of sodium hydroxide. This proved to be much more soluble than 2-oxy-6-aminopyrimidine (cytosine) (VIII).[1] When 2-oxy-6-ethylaminopyrimidine was nitrated in the manner described below, a quantitative yield of 2-oxy-5-nitro-6-ethylaminopyrimidine (VI) was obtained. This compound was reduced to 2-oxy-5-amino-6-ethylaminopyrimidine (V) by means of freshly precipitated ferrous hydroxide. The yield was not quantitative although it probably exceeded 75 per cent of theory. A very soluble by-product was formed and it was difficult to separate all of the diaminopyrimidine from this compound. The conversion of 2-oxy-5-amino-6-ethylaminopyrimidine to 2,8-dioxy-9-ethylpurine (IV) by heating with urea took place very smoothly.

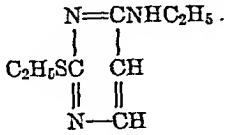
These researches will be continued.



[1] Wheeler and Johnson: *Amer. Chem. Journ.*, xxix, p. 498, 1903.

EXPERIMENTAL PART.

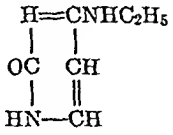
2-Ethylmercapto-6-ethylaminopyrimidine.



A mixture of 10 grams of 2-ethylmercapto-6-chlorpyrimidine[7] and 18 grams of 33 per cent, aqueous ethylamine was heated in a sealed tube at 100°C. for 24 hours. The resulting heavy oil was washed with water to remove salts and was then dissolved in ether and the solution was dried over calcium chloride. After drying, the ether was distilled off and the oil was purified by distilling it under diminished pressure. It boiled at 199.5°C. at a pressure of 11 mm. It was practically pure before it was distilled. The yield was almost quantitative.

	Calculated for $C_8H_{13}N_3S$:	Found:
N.....	22.95	23.02

2-Oxy-6-ethylaminopyrimidine.



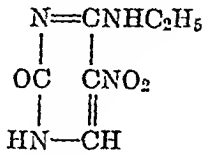
Six grams of 2-ethylmercapto-6-ethylaminopyrimidine were boiled with concentrated hydrochloric acid until the mercaptan was all expelled, and the resulting solution was then evaporated to dryness on a steam bath. The residue was treated with a slight excess of sodium hydroxide and again evaporated to dryness. The free base was extracted from this residue by means of hot, absolute alcohol. The extract was concentrated and set aside to cool. The base crystallized out in the form of bluntly pointed prisms. These crystals were further purified by recrystallizing once from water and once from alcohol. They melted to an oil at 218°C. They were easily soluble in hot water or alcohol and insoluble in benzene

[7] Wheeler and Johnson: *loc. cit.*

or ether. They formed a hydrochloride from which all of the hydrochloric acid was not removed by evaporating with an excess of ammonia. When the above procedure was used in isolating the base the yield was quantitative.

	Calculated for $C_6H_9ON_3$:	Found:
N	30.21	30.19

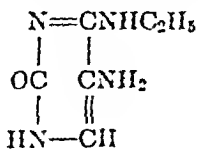
2-Oxy-5-nitro-6-ethylaminopyrimidine.



Four grams of 2-oxy-6-ethylaminopyrimidine were dissolved in 8 cc. of concentrated sulphuric acid, and 3 cc. of fuming nitric acid, of specific gravity 1.5, were added gradually. The temperature was kept below 70°C. After the mixture had stood at room temperature for fifteen minutes, it was poured on cracked ice and the acids were neutralized with ammonia. This was accomplished by adding a slight excess of ammonia and then acidifying with a slight excess of acetic acid, because the nitro compound forms a soluble ammonium salt. The nitro pyrimidine separated in the form of a bulky crystalline precipitate. The yield was quantitative. The portion used for analysis was recrystallized from water and was thus obtained in the form of acicular prisms. These decomposed at 275°C. without melting. They were soluble in dilute alkalies, moderately soluble in hot water, slightly soluble in hot alcohol, but insoluble in benzene or ether.

	Calculated for $C_6H_7O_3N_4$:	Found:
N	30.43	30.41

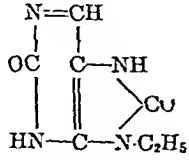
2-Oxy-5-amino-6-ethylaminopyrimidine.



Four grams of 2-oxy-5-nitro-6-ethylaminopyrimidine were dissolved in a mixture of 50 cc. of concentrated ammonia and 75 cc. of water by warming gently. This solution was cooled to room temperature and a solution of 42 grams of crystallized ferrous sulphate was added gradually. The nitro pyrimidine was reduced rapidly, the reaction being exothermic. Forty-eight grams of crystallized barium hydroxide, dissolved in water, were added to precipitate the sulphate, and the excess of baryta was removed by means of ammonium carbonate. After this mixture had stood at room temperature for a half hour, the precipitates were filtered off and the filtrate was concentrated to a small volume and clarified with blood coal. On concentrating further and cooling, the diamino pyrimidine crystallized in beautiful, short, stout prisms which were nearly colorless. These crystals decomposed without melting at 240°C. They were very soluble in hot water and crystallized well from concentrated solutions, on cooling slowly. They were slightly soluble in hot alcohol and insoluble in benzene or ether. An ammoniacal solution of this pyrimidine gave a deep blue color on the addition of a solution of phosphotungstic acid.[8] In ammoniacal solution it formed a crystalline silver salt which did not darken until the mother liquor containing it was heated. A picrate was obtained from concentrated solutions but this salt was too soluble to be of value in identifying the pyrimidine.

	Calculated for $C_6H_{10}ON_4$:	Found:
N	36.36	36.30

2,8-Dioxy-9-ethylpurine.



Three grams of 2-oxy-5-amino-6-ethylaminopyrimidine and three grams of urea were pulverized together and the mixture was heated in an oil bath at 170–180°C. for an hour. The mass melted and the escaping ammonia produced considerable effervescence. The re-

[8] Johnson and Johns: *Journ. Amer. Chem. Soc.*, xxxvi, p. 970, 1914.

action-product was a hard cake. This was cooled and dissolved in hot dilute ammonia and the solution was clarified with blood coal. This treatment left an almost colorless solution from which most of the ammonia was removed by heating on a steam bath. On adding a slight excess of acetic acid and cooling, a large portion of the purine separated in a crystalline form. A second crop of crystals was obtained by concentrating the mother liquor. The yield was three grams. The purine was purified by recrystallization from water. The crystals consisted of small anhydrous prisms which decomposed, without melting, when heated above 300°C. They dissolved easily in hot, dilute ammonia and moderately in hot water. They were slightly soluble in hot alcohol but did not dissolve in benzene or ether.

	Calculated for $C_7H_4O_2N_4$:	Found:
C	46.66	46.90
H	4.44	4.38
N	31.11	31.13

UREA AND TOTAL NON-PROTEIN NITROGEN IN NORMAL HUMAN BLOOD: RELATION OF THEIR CONCENTRATION TO RATE OF ELIMINATION.

By FRANKLIN C. McLEAN and LAURENCE SELLING.

(From the Laboratory of Pharmacology, Medical Department, University of Oregon.)

(Received for publication, July 7, 1914.)

Folin and his collaborators have recently described extremely accurate and comparatively simple new methods for the determination of the various nitrogenous constituents of the blood and urine. By the use of these methods we may expect a material increase in our knowledge of nitrogen metabolism and excretion in health and disease. For the application of these methods to the study of diseased conditions, however, it is necessary that the normal variation and relationships be first firmly established. It is the purpose of this paper to present the results of experiments on normal adults, having especially in mind the normal urea content of blood and urine.

Folin and Denis[1] report the results of blood analyses made in sixteen normal individuals. In these sixteen cases the total non-protein nitrogen varied only between 22 and 26 mgm. and the urea nitrogen between 11 and 13 mgm. per 100 grams of blood. They concluded that the "perfectly normal kidney" maintains a constant level of non-protein nitrogen and urea in the blood. All of these sixteen experiments were made in the morning, three to six hours after breakfast. They found, however, that when they made analyses of the blood from any kind of clinical material they found much larger amounts, even though there was no other evidence of kidney disturbance. Others working with the same methods, notably Farr and Austin[2] and Agnew,[3]

[1] This *Journal*, xiv, p. 29, 1913.

[2] *Journ. of Exp. Med.*, xviii, No. 3, 1913.

[3] *Arch. of Int. Med.*, xiii, p. 485, 1914.

have found that higher values are not necessarily associated with renal disturbance. None of these observers has compared the concentration of these substances in the blood with their elimination in the urine.

Recently Marshall and Davis,[4] working on animals and using the methods recently described by Marshall have found that the rate of excretion of urea in normal animals is directly proportional to the concentration in the blood. They also found that the elimination in the urine may be retarded by dehydration of the organism.

A number of French writers, notably Ambard, Widal and Achard[5] have compared the concentration and amount of urea excreted in the urine with its concentration in the blood, and have apparently established a close parallelism between the concentration of urea in the blood and the amount excreted in the urine. Ambard formulated the following laws as the result of his determinations under normal conditions: 1. When the concentration of urea in the urine is constant, the quantity of urea excreted in the urine varies proportionately to the square of the concentration of the urea in the blood. 2. When the concentration of urea in the blood remains constant the quantity excreted in the urine varies inversely as the square root of the concentration in the urine. As a resultant of laws 1 and 2, corrected by the factors of the weight of the individual and the average concentration of urea in the urine, which he determined as 25 grams per liter, he formulated the following coefficient:

$$K(\text{constant}) = \frac{Ur}{\sqrt{D \times \frac{70}{P}\sqrt{\frac{C}{25}}}}$$

Ur = Urea per liter of blood, in grams.
D = Urea in urine in twenty-four hours, in grams.
P = Weight of patient, in kilos.
C = Concentration, or grams urea per liter of urine.

Ambard, Widal and others found this coefficient to be a constant in normal individuals, varying only between 0.06 and 0.07 under

[4] This *Journal*, xviii, p. 53, 1914.

[5] Ambard: *Compt. rend. soc. biol.*, 1910, Dec. 3, p. 506; complete literature in Achard: *Le rôle de l'urée en pathologie*, Paris, 1912.

varying conditions of diet, etc. They found, however, an enormous increase in the value of the coefficient in cases of nephritis, associated with a relative increase in the concentration in the blood and a decrease in the elimination. Their studies have been extended to large numbers of clinical cases, and they have come to far-reaching conclusions regarding the value of such studies in the diagnosis and prognosis of various conditions associated with deficient elimination. The results and conclusions of the French school have been criticised by American writers on the ground that they used an inaccurate method for the determination of urea.

Our work has been undertaken to correlate the results obtained by American workers with those obtained by the French writers. To carry out this purpose we have made simultaneous estimations of the total non-protein nitrogen and urea nitrogen of the blood and of the total nitrogen and urea nitrogen of the urine. Students, technicians, instructors, etc., were utilized after their urines had been found to be free of evidence of renal disturbance. The results may be accepted as from perfectly normal adults, between the ages of 20 and 40. The examinations were made at various times during the day, and particular care was taken to obtain the maximum normal variations in diet, water intake, etc., and to make the examinations at varying times with regard to meals, etc. The results of the experiments made are herewith tabulated. In the first series of experiments made, tabulated in Table II, a twenty-four-hour period was used, the total urine for that period being collected and the blood withdrawn once during the day. The results by this method were soon seen to be inaccurate since the urea of the blood fluctuates from hour to hour and the urea content of the blood at the time of examination did not always represent an average of the concentration during the twenty-four-hour period. In our larger series of examinations, covering twenty-six experiments, a shorter period, usually one hour, was used and the blood collected at the middle of the period. By this method the amount of urea in the blood may be safely assumed to represent the average concentration for the period in case no food, etc., has been taken during the period. It is possible that the results might have been somewhat more constant had catherization been performed in each case.

TABLE I. *Results in normal individuals: length of*

SUBJECT	WEIGHT	DATE	PERIOD	BLOOD (mgm. per 100 cc.) Non-protein N	Urea N	Urea	Amount cc. Period	24 hrs.
	kilos							
F. C. M...	77.2	IV, 29	9.15–10.15	30	17.0	36.3	40	960
	77.2	V, 6	9.15–10.15	30	12.0	25.6	177	4248
	77.2	V, 11	2.45– 3.45	28	15.0	32.1	83	2000
	77.2	V, 18	2.45– 3.45	27	18.0	38.5	40	960
	77.2	V, 21	11.00–12.00	27	15.0	32.1	34	816
	77.2	V, 25	3.30– 5.30	44	24.0	51.3	128	1536
	77.2	V, 28	2.45– 3.45	28	19.0	40.6	101	2424
	77.2	VI, 1	10.00–11.00	23	14.0	29.9	30	720
R. A. S....	70.0	V, 5	4.00– 5.00	36	15.0	32.1	155	3720
	70.0	V, 12	3.30– 4.30	29	17.0	36.3	38	912
	70.0	V, 25	3.45– 4.45	31	20.0	42.8	78	1872
L. S.....	55.0	V, 19	11.15–12.15	34	18.0	38.5	40	960
	55.0	V, 19	3.12– 4.47	33	16.5	35.3	70	1056
	55.0	V, 23	7.20– 9.20 p.m.	35	20.0	42.8	98	1176
A. T.....	70.0	V, 15	4.00– 5.00	33	21.0	44.9	50	1200
	70.0	V, 21	10.45–11.45	31	20.0	42.8	57	1368
	70.0	V, 28	2.30– 3.30	35	24.0	51.3	142	3400
W. C. M..	61.0	V, 11	4.00– 5.00	34	23.0	49.2	13	312
	61.0	V, 18	2.00– 3.00	28	18.0	38.5	117	2800
C. A. L...	84.0	V, 12	3.30– 4.30	30	16.0	34.2	42	1000
	84.0	V, 25	4.00– 5.00	30	27.0	57.7	46	1100
A. A. G.....	64.0	V, 15	4.00– 5.00	24	16.0	34.2	145	3500
H. K. A...	60.0	V, 22	7.45– 8.45 p.m.	30	22.0	47.0	37	988
	60.0	VI, 4	4.00– 6.00	28	17.0	36.3	92	1100
	60.0	VI, 5	9.00–11.30	23	15.0	32.1	250	2400
R. E. P.....	66.0	V, 4	2.00– 3.00	29	18.0	38.5	40	960

period, one to two and one-half hours.

URINE					Ambard's coeffic.	REMARKS
Grams in 24 hours			Grams per liter			
Total N	Urea N	Urea	Urea N	Urea		
	13.7	29.3	14.6	31.2	0.065	
16.1	14.6	31.2	3.6	8.0	0.064	
14.2	13.0	27.8	6.5	13.9	0.078	
11.1	10.3	22.0	10.8	23.1	0.087	
	9.4	20.1	11.6	24.8	0.075	
25.2	24.6	52.6	16.4	35.1	0.068	45 minutes after urea, 10 gm.
35.8	29.6	63.3	11.8	25.2	0.053	3 hours after urea, 10 gm.
7.9	6.9	14.7	9.7	20.7	0.085	3 days protein-poor diet.
15.5	13.7	29.3	3.8	8.1	0.078	
9.8	8.8	18.8	9.7	20.7	0.087	
21.6	20.1	43.0	10.8	23.1	0.065	1 hour after urea, 5 gm.
11.6	10.5	22.4	11.0	23.5	0.072	
13.3	12.3	26.3	11.6	24.8	0.061	
11.8	11.1	23.7	9.6	20.9	0.081	After heavy dinner.
14.4	13.4	28.6	11.3	24.1	0.084	
13.9	13.1	28.0	9.6	20.9	0.084	
40.8	38.0	81.3	11.2	23.9	0.059	90 minutes after urea, 10 gm.
5.3	4.5	9.6	14.5	31.0	0.140	Note small amount of urine.
16.2	15.6	33.3	5.6	11.9	0.070	Drinking sufficient water.
10.0	9.2	19.6	9.2	19.6	0.089	
17.6	16.3	34.8	14.8	31.6	0.101	1 hour after urea, 10 gm.
22.4	19.6	41.9	5.6	11.9	0.073	
	11.4	24.3	11.6	24.8	0.088	After heavy dinner.
12.6	11.2	23.9	10.2	21.8	0.071	
10.8	9.6	20.9	4.0	8.2	0.085	
11.6	10.1	21.6	10.6	22.6	0.082	

All of the determinations were made by the methods described by Folin and his collaborators.[6] Compressed air under high pressure was used for driving off the ammonia, and this seems to be necessary for accurate results, as we have found the vacuum pump to be entirely unreliable. All colorimetric readings were made with a new model Kennicott-Sargent colorimeter.

In Table I we have tabulated the results obtained in the shorter periods. In this table urea nitrogen has been calculated to urea, and the results obtained from the urine have been computed to twenty-four hours. Ambard's coefficient has been applied in each case, according to the formula given above. This series includes the results of four experiments following the ingestion of urea.

In Table II are the results of the twenty-four-hour periods, the urea being expressed only in terms of urea nitrogen for the sake of brevity.

TABLE II.

Normal individuals, twenty-four-hour periods.

Subject	Weight	Date	Blood (mgm. per 100 cc.)		Urine				Ambard's coeffic.
			Non-protein N	Urea N	In 24 hours			Per liter urea N	
					Amt.	Total N	Urea N		
	kilos	April			*cc.*	*grams*	*grams*	*grams*	
F. C. M.	77.2	7	26	10.0	860	8.8	8.4	9.8	0.069
L. S.	55.0	7	26	15.0	1330	8.7	8.2	6.2	0.079
C. A. L.	84.0	7	26	20.0	1250	11.7	11.5	9.2	0.100
H. K. A.	60.0	8	25	14.0	700	7.3	6.8	9.8	0.074
D. A. P.	60.0	8	26	10.5	1900	8.3	7.4	3.9	0.068
R. A. S.	70.0	10	23	11.0	1150	12.6	11.2	9.8	0.050
W. C. M.	61.0	10	26	15.0	570	4.6	3.9	6.9	0.118
A. T.	70.0	12	29	19.0	1520	17.1	15.8	10.4	0.071
C. C.	72.0	18	28	17.0	2000	13.4	11.8	5.8	0.087
H. U.	45.0	18	29	18.0	750	7.7	7.0	9.4	0.084

[6] Folin: this *Journal*, xi, p. 507, 1912; Folin and Denis: *ibid.*, p. 527; Folin and Farmer: *ibid.*, p. 493.

Discussion.

From the above tables it will be seen that the urea nitrogen of the blood in normal individuals (not including those following the ingestion of urea) varies in our series from 10 to 23 mgm. per 100 cc. of blood, while the total non-protein nitrogen varies from 23 to 36 mgm. in the same individuals. Applying Ambard's coefficient to the same figures, we find that it varies in the shorter periods only between 0.061 and 0.089. The only exception is W. C. M., who twice showed a high coefficient, associated with a deficient elimination of water. His coefficient fell to within normal limits when sufficient water was taken, and his case corroborates the finding of Marshall and Davis on animals that dehydration of the organism caused a retardation of the elimination of urea. In the remainder of the series, under wide variations of diet, etc., Ambard's coefficient remained remarkably constant, though the concentration of urea in the blood varied within wide limits. One series includes individuals excreting from 7.9 to 22.4 grams of nitrogen daily, and passing from 700 to 3720 cc. of urine in the twenty-four hours, which would represent the average limits of normal variations.

The results following the ingestion of urea are interesting in that they show in general a normal coefficient with an enormous rate of urea excretion. In one case a coefficient of 0.101 was obtained, and this was attributed to the rapid absorption of urea during the period, causing a higher relative concentration in the blood.

In order to show the effects of impaired elimination of urea on the value of the coefficient, without necessarily a high absolute amount of urea in the blood Table III is appended. The results of these cases with others studied will form the basis of a later paper, but this table when compared with Table I shows clearly that the absolute amount of urea in the blood cannot be taken as evidence of "nitrogen retention" since the concentration of urea in the blood in these cases (all cases of frank nephritis with other evidence of impaired elimination), does not differ materially from that in many normals.

TABLE III (*for comparison*).

All positive nephritics. Length of periods, one to twenty-four hours.

SUBJECT	WEIGHT	DATE	BLOOD (mgm. per 100 cc.)		URINE				
					In 24 hours			Per liter urea N	Ambard's coeffic.
			N	Urea N	Amt.	N	Urea N		
					cc.	*grams*	*grams*	*grams*	
B.....	70	V, 23	50	29	1250	10.3	9.6	7.6	0.150
A.....	70	V, 30	41	25	1500	12.7	11.9	8.0	0.116
J.....	90	V, 21	43	27	1380	13.2	12.4	9.0	0.135
E.....	52	V, 12	31	24	744	9.4	8.8	11.8	0.127
R.....	66	IV, 7	25	15	1000	6.6	5.8	5.8	0.105
B.....	70	IV, 23	36	19	860	8.9	8.0	9.4	0.103
H.....	70	VI, 2	40	26	720	9.0	7.6	10.6	0.141

CONCLUSIONS.

1. The concentration of total non-protein nitrogen and of urea in normal human blood is not constant, but varies within wide limits according to various factors of diet, amount of fluid ingested, etc.

2. There is a close parallelism between the concentration of urea in the blood and the amount excreted in the urine, in normal individuals under average conditions. The elimination of urea may be retarded by "dehydration of the organism," as shown in animals by Marshall and Davis.

3. Ambard's coefficient, when computed from results obtained by the accurate methods of Folin and his collaborators, varies in normal individuals only between comparatively narrow limits, and may be regarded as a constant.

4. A concentration of urea nitrogen as high as 22 mgm. per 100 cc. of blood does not necessarily indicate any disturbance in elimination of urea unless associated with a relative decrease in the amount excreted. Ambard's coefficient may be used for the purpose of making the necessary comparison.

5. Ingestion of urea does not materially alter the value of Ambard's coefficient, providing sufficient time is allowed for absorption before examination is made.

THE ELIMINATION OF PHENOLSULPHONEPHTHALEIN IN ACUTE AND CHRONIC TARTRATE NEPHRITIS.

By FRANK P. UNDERHILL and NORMAN R. BLATHERWICK.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, Conn.)

(Received for publication, July 8, 1914.)

The elimination of phenolsulphonephthalein in experimental nephritis produced by a variety of agents has been studied by Eisenbrey,[1] who found that the excretion of the dye is decreased in so-called spontaneous nephritis, and in the nephritis induced by potassium chromate, cantharidin, uranium nitrate, diphtheria toxin and arsenic, and also in those lesions caused by snake venom, hemolytic serum, prolonged renal anemia and extensive reduction of the kidney substance. On the other hand, its elimination is increased rather than diminished, in the presence of the renal lesions provoked by nephrotoxic serum. For this result no explanation is at hand.

In another study Austin and Eisenbrey[2] have shown that the decrease in the elimination of phenolsulphonephthalein, which occurs in uranium, chromate, and cantharidin nephritides, and which, in a general way, is proportional to the dose of the poison, bears no constant relation to the changes in the nitrogen or chloride elimination.

In previous communications[3] it has been demonstrated that tartrates subcutaneously injected into rabbits produce a nephritis which in general is a clear-cut tubular nephritis. Pearce and Ringer[4] employing dogs, and Karsner and Denis[5] using cats have confirmed the production of a severe nephritis with tartrates but

[1] Eisenbrey: *Journ. of Exp. Med.*, xiv, p. 462, 1911.

[2] Austin and Eisenberg: *Journ. of Exp. Med.*, xiv, p. 366, 1911.

[3] Underhill: This *Journal*, xii, p. 115, 1912; also Underhill, Wells and Goldschmidt: *Journ. of Exp. Med.*, xviii, p. 317, p. 322 and p. 347, 1913.

[4] Pearce and Ringer: *Journ. of Med. Research*, N. S., xxiv, p. 57, 1913.

[5] Karsner and Denis: *Journ. of Exp. Med.*, xix, p. 259, 1914.

affirm that in some instances at least the glomerular mechanism is involved as well as the convoluted tubules. Inasmuch as our conclusions were drawn from experiments made for the most part upon rabbits, there is no necessary contradiction in the results of the above mentioned investigators and our own. It should be stated, however, that in the few experiments upon dogs carried out by us the form of nephritis produced by tartrate was of the tubular type.

Since the experimental nephritides studied by Eisenbrey are usually of the mixed type, although one part of the renal mechanism may be more severely injured than the other in different animals, it seemed desirable to study the elimination of phenolsulphonephthalein in tartrate nephritis, where it must be accepted at least that the tubular portion of the renal mechanism is the part more often showing significant injury. In those animals which survived the injection of tartrate the elimination of the dye was followed first during the acute stage and also at later periods when sufficient time had elapsed for the injury to take on the chronic form.

Methods. Our experiments were confined to rabbits subjected to a period of starvation since food is refused after the administration of tartrate. Free access to water was allowed during the latter portion of each day. The tartrate given was of the racemic form,[6] and was purposely administered in relatively small doses so as to allow the animals to live for a reasonable length of time. In beginning an experiment tartrate was not given until the third day of starvation. On the second day of inanition the elimination of the dye was determined in one- and two-hour periods and the figures thus obtained were regarded as the normal for an individual rabbit. Ten minutes previous to the subcutaneous injection of 1 cc. of phenolsulphonephthalein solution (6 mgms. per cubic centimeter) diluted to 2 cc. with water, the animals received 50 cc. of water by stomach tube. Urine was obtained by compression of the abdominal wall over the bladder. The experi-

[6] In this connection it may be well to point out that the optical activity of the tartaric acid employed apparently plays little or no rôle in influencing the production of nephritis for the renal injury is so far as one may judge of the same type and degree with dextrorotatory tartaric acid as with the racemic. This fact is further emphasized by the work of Pearce and Ringer, Karsner and Denis who used *d*-tartaric acid, and also by that of Salant and Smith (*Proc. Soc. Exp. Biol. and Med.*, x, p. 170, 1913), who employed the *d*- and *l*-forms of tartaric acid.

ment was begun with the bladder empty. The tartrate was usually injected about 10.30 a.m. and the first subsequent test for phenolsulphonephthalein elimination was carried through on the same day, the dye being injected at 2 p.m. The coloring matter excreted was estimated by means of an Autenrieth-Königsberger colorimeter. In addition to the determination of the dye excretion during the two one-hour periods estimations were made in the twenty-four hours urine specimens for total nitrogen and hydrogen ion concentration.[7] At the end of each test period the animals were placed in large pens and well fed until the body weight had regained its initial value. They were then subjected to a second test period without food. In a similar manner a third period was carried through. The kidneys of those animals surviving the entire period of experimentation were preserved in Zenker's fluid and in formalin and sent to Prof. H. Gideon Wells of the University of Chicago to whom we are indebted for the histological reports accompanying the tables presented below.

EXPERIMENTAL.

The results of the influence of sodium tartrate upon the elimination of phenolsulphonephthalein may be seen from the data given in tables 1 to 6 inclusive. Table 7 contains the results obtained with a rabbit serving as a control, no tartrate being administered.

Rabbit 1. In the first period it is apparent that the elimination of the dye is markedly retarded when compared to the normal excretion. A certain degree of improvement is noticeable during the second period—ten days later. The rapidity of dye excretion was not significantly altered throughout the third period. From the histological report it is also apparent that there is in this animal a considerable degree of injury to the glomerulus.

Rabbit 2. The injury sustained as a result of tartrate injection was too severe for this animal to survive beyond a period of four days during which interval there was a complete inhibition of the dye elimination. No histological study of the kidneys was made. Attention is called to the fact that although the

[7] Henderson, L. J.: This *Journal*, xiii, p. 393, 1913.

dye excretion was greatly retarded there is to be noted a *considerable excretion of total nitrogen daily.*

Rabbit 3. With this animal tartrate did not produce a profound renal lesion if one may judge from the rapidity of phenolsulphonephthalein elimination. On the other hand, in the period of our investigation no definite approach to the normal excretion was manifested. On the first day of tartrate influence the total nitrogen excretion was noticeably diminished. The histological report confirms the view that the kidneys suffered only a slight injury from the administration of tartrate.

Rabbit 4. Although the histology of the kidney of Rabbit 4 differs little from that of Rabbit 3 there is a marked difference in the excretion of the dye and also of the total nitrogen and in neither respect did the kidneys regain the normal secreting power.

Rabbit 5. From the figures for the "normal" elimination of the dye it is evident that this animal was probably in a condition of spontaneous nephritis. After an initial marked fall in secretory power due to tartrate injection this power of the renal organs increased to a certain extent and then remained stationary even after an appreciable interval of time.

Rabbit 6. In this animal the injection of tartrate shows only a slight detrimental influence upon the elimination of the dye and in accord with this there is to be noted from the histological report that the kidneys were not severely injured.

Rabbit 7—no tartrate given. This animal was selected to serve as a control being subjected to conditions precisely similar to those of the other rabbits except that no tartrate was injected. Both from the histological report and from the rate of dye elimination it is evident that this rabbit did not possess normal kidneys.

DISCUSSION.

From a study of the data presented it may be seen that tartrate nephritis causes a markedly diminished rate of phenolsulphonephthalein elimination and that in general when the animal survives sufficiently long an unmistakable degree of improvement may be noticed. This improvement increases up to a certain point and the condition of the kidney then remains stationary,

the normal rate of elimination not being regained during the period of observation set for this investigation.

A survey of the histological reports presents an interesting feature in that in most instances the glomerulus is involved as well as the tubule, results which are in entire disagreement with our previous experience. An explanation for this discrepancy lies perhaps in the length of time the rabbits lived after tartrate injection. It is possible that in the chronic state the injury may extend to the glomerulus, the initial injury being exerted upon the tubule. In previous work kidneys examined by us were taken at intervals relatively short compared with those in the present work.

Little change is to be observed in the acidity of the urine as indicated by the hydrogen ion concentration.

In certain of these experiments in which little or no dye was eliminated during the two one-hour periods it was observed that the dye never appeared in the urine. Investigation of the point has demonstrated that in the tartrate animals and also in the control the dye may be excreted through the feces. With other animals the kidneys were excluded from the circulation by first ligating the renal vessels and then passing a mass ligature around the kidney. Dye injected into such an animal appeared in the feces, apparently by way of the bile, inasmuch as the dye was present in this secretion.

From a study of the character of the daily nitrogen output a point of interest is evident. The nitrogen excretion for the second day of starvation may be taken fairly as a standard for the nitrogen output of the succeeding days according to our experience with fasting rabbits. Accepting this standard and comparing it with the average output for the succeeding experimental days it is apparent that in general little difference is to be observed. This fact is made more obvious by the data presented in table 8. Expressed differently, the total nitrogen elimination during the periods of observation is little changed from the normal in spite of the fact that the rate of phenolsulphonephthalein elimination shows a marked diminution. In the acute stage of tartrate nephritis the rate of dye excretion and daily nitrogen output run parallel. After the condition has become chronic, however, there is to be observed a noticeable divergence in the

output of dye and nitrogen. This observation leads to the query, "Does the rate of phenolsulphonephthalein elimination as truly represent the *actual* efficiency as a study of the excretion of total nitrogen?" From the experiments reported above one sees that in the *chronic* condition although the *rate* of elimination, as indicated by phenolsulphonephthalein, is certainly decreased *total* renal efficiency, as judged by nitrogen output, is about normal. There may be temporary retention in the blood of urinary nitrogenous substances but over an extended period the usual absolute quantity of these constituents may be removed. Compensatory function ensues.

If these ideas are applied to certain types of chronic nephritis in man a possible explanation is found for the fact that these cases do not sooner terminate fatally. The phenolsulphonephthalein test is undoubtedly a good indicator of a changed *rate* of elimination but it does not necessarily show that the *total work* of the kidney over an extended period at least is insufficient for the elimination of excretory substances to the degree requisite for the maintenance of nutritional rhythm. If the number of efficient kidney cells is notably decreased it is evident that in order to have a certain amount of waste excreted the normal cells must work more constantly than if none of the cells were incompetent. So long as the total waste is eliminated does it necessarily mean inefficient kidneys even though the *rate* of elimination is slow?

SUMMARY.

The rate of phenolsulphonephthalein elimination may be markedly decreased during the acute stage of tartrate nephritis.

In the chronic condition the excretion of dye improves to a certain extent but does not regain the normal at least during the period of observation of this investigation.

In certain animals of this series of experiments the glomerulus was injured as well as the tubule. It is possible that the state of chronicity of the experimental nephritis may be responsible for this discrepancy between our present and former results.

During tartrate nephritis hydrogen ion concentration varies little from that observed in starvation previous to tartrate injection.

When excretion by the kidney is prevented, phenolsulphonephthalein is eliminated in the feces, through the bile.

After tartrate injection the average nitrogen excretion is little changed from that observed previous to the tartrate administration. Although the *rate* of elimination is undoubtedly diminished the *total amount* of waste excreted is unchanged. The rate of elimination therefore may not mean necessarily that the renal organs are not efficient.

It is suggested that this observation may explain the longevity of persons suffering from certain types of human nephritis.

TABLE 1.

Rabbit 1. Body weight, 3400 grams. Moderate nephritis.

DATE	URINE									REMARKS
	Volume	(+H)	Total N	Phenolsulphonephthalein elimination						
				1st hour		2nd hour		Total 2 hours		
				Urine volume	Dye recovered	Urine volume	Dye recovered	Urine volume	Dye recovered	
	cc.		gms.	cc.	per cent	cc.	per cent	cc.	per cent	
Period 1.										
Feb. 8	250	6.50	1.15							First day of fast. No protein or casts.
9	125	5.70	0.96	19	54	20	37	39	91	
10	7		0.011	4	Faint trace	2	Faint trace	6	Trace	0.85 gram tartaric acid neutralized with NaOH.
11	164	5.10	0.607	1 drop	Faint trace	0		1 drop	Trace	Cloudy. Colorless. Much protein. Many casts.
12	184	4.90	1.77	18	Distinct trace	10	Distinct trace	28	Trace	Much protein. Many casts.
13	112	5.30	1.27	8	Heavy trace	3	Heavy trace	11	Trace	Much protein. Many casts.
14				10	10	6	15	16	25	

Period 2.

23	120	6.00	1.06	21	17	29	17	50	34	Slightly cloudy. Protein present.
24	81	5.10	0.86	20	25	23	24	43	49	Slightly cloudy. Protein present.
25				18	27	25	25	43	52	Slightly cloudy. Protein present.

Period 3.

Mar. 5	84	5.70	0.91	23	29	18	23	41	52	Slightly cloudy. Protein present.
6	82	5.30	1.02	15	32	10	18	25	50	Slightly cloudy. Protein present.
7				9	36	10	22	19	58	Animal killed. Kidneys pale and somewhat larger than usual.

Histological report. Rabbit 1. Kidney. There is a diffuse but slight increase in the interstitial tissue of the cortex, especially just beneath the capsule, which shows little active proliferation, except about a few of the glomeruli which are surrounded by a zone of fibroblasts and lymphoid cells. The tubules in many instances show a hyaline necrosis, often with complete occlusion of the lumen by desquamated material. On account of poor fixation so large a proportion of the tubules of the cortex have dropped out of all the sections that the exact portions of the tubules most affected cannot be determined, but in many instances it seems to be the so-called "third portion" of the convoluted tubules. In many of the degenerated tubules there remains a flattened epithelium, often in layers like a stratified epithelium compressed by the hyaline material filling the tubules. There are also many hyaline casts of typical character. Many of the portions of the convoluted tubules closest to the medulla show vacuolization and coagulation necrosis. The collecting tubules seem normal. In addition to the pericapsular changes, many of the glomeruli are infiltrated with round cells, some being replaced completely by such cells; in others the proliferation of the capsule has caused marked compression of the glomerule. Mostly they are swollen and hyperemic, but some are shrunken. No hyaline or fibroid glomerules are found.

This kidney shows a subacute glomerule nephritis, apparently of some weeks or months duration, with the acute toxic destruction (of moderate degree) superadded.

TABLE 2.

*Rabbit 2. Body weight, 2300 grams. Severe nephritis.**

DATE	URINE									REMARKS
	Volume	$(\overset{+}{H})$	Total N	Phenosulphonephthalein elimination						
				1st hour		2nd hour		Total, 2 hours		
				Urine volume	Dye recovered	Urine volume	Dye recovered	Urine volume	Dye recovered	
	cc.		*grams*	*cc.*	*per cent*	*cc.*	*per cent*	*cc.*	*per cent*	
Feb. 8	260	6.90	1.39							First fasting day. No protein or casts.
9	120	6.00	0.97	22	50	29	38	51	88	
10	148	5.10	0.19	7	Distinct trace	3	Distinct trace	10	Trace	0.58 gram tartaric acid neutralized with NaOH injected at 10.30.
11	142	4.90	0.85	5	Distinct trace	5	Distinct trace	10	Trace	Colorless. Much protein. Many casts.
12	158	5.10	1.80	7	Distinct trace	11	Distinct trace	18	Trace	Much protein. Many casts.
13	79		0.67	7	Heavy trace	7	Heavy trace	14	Trace	Much protein. Many casts. Died during the night.

*Kidney sections were not made.

TABLE 3.

Rabbit 3. Body weight, 2900 grams. Slight nephritis.

DATE	URINE									REMARKS
	Volume	(+/H)	Total N	Phenolsulphonephthalein elimination						
				1st hour		2nd hour		Total, 3 hours		
				Urine volume	Dye recovered	Urine volume	Dye recovered	Urine volume	Dye recovered	
	cc.		*grams*	*cc.*	*per cent*	*cc.*	*per cent*	*cc.*	*per cent*	
					Period 1.					
Feb. 8	285	6.15	1.01							First day of fast. Trace protein.
9	123	5.50	1.22	20	40	14	31	34	71	
10	80	5.50	0.54	6	41	3	23	9	64	0.73 grams tartaric acid neutralized with NaOH injected at 10.30.
11	92	5.70	1.37	13	30	5	25	18	55	Clear. Colorless. Little protein. Few casts.
12	76	5.70	1.06	16	50	16	25	32	75	Slightly cloudy. Little protein. Few casts.
13	116	5.70	1.20	18	47	8	17	26	64	Cloudy. Little protein. Few casts.
14				5	30	35	45	40	75	
					Period 2.					
23	60	6.00	0.93	8	30	6	17	14	47	Slightly cloudy. Trace protein.
24	85	6.00	1.23	15	36	34	43	49	79	Slightly cloudy. Trace protein.
25				7	34	6	15	13	49	

TABLE 3—Continued.

DATE	URINE									REMARKS
	Volume	(+/H)	Total N	Phenolsulphonephthalein elimination						
				1st hour		2nd hour		Total, 2 hours		
				Urine volume	Dye recovered	Urine volume	Dye recovered	Urine volume	Dye recovered	
	cc.		grams	cc.	per cent	cc.	per cent	cc.	per cent	
Period 3.										
Mar. 5	47	5.70	1 00	18	32	38	27	56	59	Clear. Trace protein.
6	82	5 30	1 44	30	40	12	18	42	58	Clear. Trace protein.
7				10	32	16	22	26	54	Animal killed. Kidneys pale and somewhat larger than normal.

Histological report. Rabbit 3. The epithelium of the convoluted tubules is much swollen, stains intensely with eosin, and looks as if partly coagulated, although there are few nuclear changes and little definite necrosis. In most of these tubules the lumen is occluded by the swelling. There is some vocuolization, especially just beneath the capsule. A few hyaline casts, evidently old, in Henle's loops. No glomerular or interstitial changes. Apparently an early stage in the tubular changes.

TABLE 4.

Rabbit 4. Body weight, 2200 grams. Moderate nephritis.

Date	Urine									Remarks
	Volume	(+/H)	Total N	Elimination of phenosulphonephthalein						
				1st hour		2nd hour		Total 2 hours		
				Urine volume	Dye recovered	Urine volume	Dye recovered	Urine volume	Dye recovered	
	cc.		grams	cc.	per cent	cc.	per cent	cc.	per cent	
						Period 1.				
Feb. 15	86	5.10	0.82							First fasting day. Slightly cloudy. No protein or casts.
16	89	5.30	1.25	13	48	16	32	29	80	Same as above.
17	12		0.04	11	0	0	0	11	0	0.56 gram tartaric neutralized with NaOH injected. Colorless. Much protein.
18	88	4.90	0.36	0	0	0	0	0	0	Milky. Much protein. Many casts. Trace of dye in feces.
19	183	4.90	1.17	10	0.5	24	0.5	43	1	Slightly cloudy, little color. Much protein. Many casts.
20	100	5.10	0.98	11	0.5	13	1.5	24	2	Cloudy. Much protein.
21				7	1.5	0	3.5	16	5	
						Period 2.				
Mar. 2	78	5.10	0.76	14	20	13	19	27	30	Protein present.
3	77	5.30	0.76	16	25	14	21	30	46	Protein present. Slightly cloudy.
4				9	22	3	8	12	30	
						Period 3.				
12	77	4.90	0.67	10	20	6	14	16	34	Protein present. Slightly cloudy.
13	78	5.10	0.66	11	23	10	22	21	45	Protein present. Slightly cloudy.
14				6	32	5	25	11	55	Animal killed and kidneys preserved.

Histological report. Rabbit 4. Practically the same as No. 3.

TABLE 5.

Rabbit 5. Body weight, 1860 grams. Moderate nephritis.

Date	Urine: Volume	Urine: (+H)	Urine: Total N	Elimination of phenolsulphonephthalein: 1st hour: Urine volume	1st hour: Dye recovered	2nd hour: Urine volume	2nd hour: Dye recovered	Total 2 hours: Urine volume	Total 2 hours: Dye recovered	Remarks
	cc.		grams	cc.	per cent	cc.	per cent	cc.	per cent	
Period 1.										
Feb. 15	46	5 50	0 74							First fasting day. No protein or casts. Slightly cloudy.
16	68	5 30	0 76	11	36	20	15	31	51	Control day for testing elimination of dye.
17	19		0 034	0	0	3	0	3	0	0.47 gram tartaric acid neutralized with NaOH injected. Colorless. Much protein.
18	123	4 90	0 58	2	Faint trace	11	Good trace	13	Good trace	Colorless. Cloudy. Much protein. Many casts.
19	106	5 50	0 92	19	7	7	2	26	9	Cloudy. Few casts. Little protein.
20	104	5 50	1 46	33	23	4	8	37	31	Cloudy. Few casts. Little protein.
21				6	32	4	12	10	44	

					Period 2.					
Mar. 2	76	5.10	0.76	13	12	8	2	21	24	Slightly cloudy. Protein present.
3	70	5.10	0.82	10	14	19	16	19	30	Slightly cloudy. Protein present.
4	95			8	20	16	17	24	37	
					Period 3.					
12	95	4.90	0.70	25	20	28	12	53	32	Slightly cloudy. Protein present.
13	67	5.10	0.76	12	22	15	12	27	34	Slightly cloudy. Protein present.

Histological report. Rabbit 5. Resembles No. 1 very closely, there being similar chronic interstitial and glomerular changes. There is perhaps a little more necrosis, of similar character to that in No. 1, and in some of the tubules there are densely packed cells with deep stained nuclei, suggestive of a regenerating process. The 1st and 2nd portions of the convoluted tubules seem to have more swollen epithelium than in No. 1, resembling more that in 3 and 4. On the whole, however, it is almost a mate for No. 1.

TABLE 6.

Rabbit 6. Body weight, 2200 grams. Slight nephritis.

DATE	URINE									REMARKS
	Volume	(+/H)	Total N	Elimination of phenolsulphonephthalein						
				1st hour		2nd hour		Total 2 hours		
				Urine volume	Dye recovered	Urine volume	Dye recovered	Urine volume	Dye recovered	
	cc.		grams	cc.	per cent	cc.	per cent	cc.	per cent	
Period 1.										
Feb. 15	41	5 85	1 16							Slightly cloudy. No protein or casts. First day of fast.
16	67	5 50	1 16	6	44	13	31	19	75	Control day for testing normal elimination of dye.
17	66	5 50	1 25	13	36	6	24	19	60	0.55 gram tartaric acid neutralized with NaOH injected.
18	49	5 70	1.01	4	38	6	28	10	66	Slightly cloudy. Trace of protein. No casts.
19	37	5 50	0 88	3	50	4	23	7	73	Slightly cloudy. Trace of protein. No casts.
20	41	5 50	0 85	2	45	4	28	6	73	Slightly cloudy. Trace of protein. No casts.
21				4	48	4	21	8	69	
Period 2.										
Mar. 2	35	5 30	0.63	6	48	5	26	11	74	Clear. Thick and jelly-like. No protein.
3	51	5 30	0 88	8	46	6	33	14	79	Slightly cloudy. No protein.
4		5 10		4	42	10	20	14	62	
Period 3.										
12	73	5 10	0 88	3	33	5	20	8	53	Clear. No protein.
13	57	5 30	0 58	6	40	16	27	22	67	Clear. No protein. Thick.
14										Animal killed and kidneys examined.

Histological report. Rabbit 6. There is considerable vacuolization of the epithelium in all parts of the convoluted tubules associated with more or less swelling but no necrosis. A few small foci of round cell interstitial infiltration are present, especially in the medulla.

TABLE 7.

Rabbit 7. Body weight, 2540 grams. Control: No tartrate given.

Date	Urine: Volume	Urine: (+/H)	Urine: Total N	Elimination of phenolsulphonephthalein: 1st hour: Urine volume	1st hour: Dye recovered	2nd hour: Urine volume	2nd hour: Dye recovered	Total 2 hours: Urine volume	Total 2 hours: Dye recovered	Remarks
	cc.		grams	cc.	per cent	cc.	per cent	cc.	per cent	
Period 1.										
Feb. 16	85	7 38	0.75							First day of fast. Clear. No protein or casts.
17	50	5 30	1.02	4	40	3	30	7	70	All the remaining urines were, as a rule clear and very thick and showed no protein or casts. A trace of dye was found in the feces.
18	44	5.70	0 89	4	36	3	24	7	60	
19	39	5.50	0.70	4	48	3	38	7	86	This rabbit received no tartaric acid.
20	36	5 50	0.92	4	40	5	22	9	62	
21				4	36	4	22	8	58	
Period 2.										
Mar. 2	43	5 30	0.82	4	37	4	24	8	61	
3	34	5 30	0.77	4	32	4	20	8	52	
4				3	42	3	31	6	73	
Period 3.										
12	66	4.90	0.80	7	41	16	28	23	69	
13	52	5.10	0.68	5	44	7	23	12	67	
14										Rabbit killed.

Histological report. Rabbit 7. Similar to No. 6, but vacuolization of cells somewhat less.

TABLE 8.

A comparison of the average daily nitrogen excretion after tartrate injection with the nitrogen output of the second day of inanition.

RABBIT	NITROGEN OUTPUT FOR SECOND DAY OF STARVATION	AVERAGE DAILY NITROGEN OUTPUT AFTER TARTRATE
	grams	*grams*
1	0.96	0.92
2	0.97	0.89
3	1.22	1.09
4	1.25	0.67
5	0.76	0.75
6	1.16	0.90
7 (control—no tartrate)	1.02	0.80*

*For a period corresponding with that of the animals receiving tartrate.

THE INHIBITION OF AUTOLYSIS BY ALCOHOL.

By H. GIDEON WELLS and GEORGE T. CALDWELL.

(From the Department of Pathology of the University of Chicago, and the Otho S. A. Sprague Memorial Institute.)

(Received for publication, July 10, 1914.)

Although alcohol is universally used as a fixative for tissues, and the experience of histologists has taught that certain percentages are necessary to produce a satisfactorily firm coagulation and permanent preservation, yet there seem to be no definite data on the exact strength of alcohol necessary to totally inhibit autolytic changes. It has been found repeatedly that tissues preserved in alcohol for many decades may retain their histological structure and staining properties almost or entirely unimpaired; on the other hand, we may often observe the formation of crystals, not only of fatty acids, but also of leucine and tyrosine, deposited on the surface of tissues kept in too weak a concentration of alcohol. As in pathological chemistry it is often necessary to preserve specimens for some time before they can be analyzed, because of the occasional and accidental occurrence of the material wanted for study, and as alcohol seems to be in most cases the only suitable preservative because it causes practically no chemical changes in the tissues, it seemed desirable to establish the exact concentration of alcohol necessary to certainly check all enzymatic disintegration of the tissues. Experience in the analysis of a number of preserved specimens had led us to suspect that the concentration of alcohol commonly used for this purpose is not always certain to prevent chemical changes taking place in the tissues.

It has been established by investigations on autolysis that sufficiently weak concentrations of alcohol permit autolysis to occur, and at the same time prevent bacterial decomposition.

The best study on this subject with which we are familiar is that of Yoshimoto,[1] who found that 5 per cent alcohol interferes less with autolysis than the commonly used chloroform water. His figures for non-coagulable nitrogen (grams N per 1000 grams liver tissue) in four series of experiments, were as follows:

	1	2	3	4
Boiled control.....	2.184	2.242	2.526	2.016
Chloroform water.....	5.376	6.533	7.504	6.720
20 per cent alcohol.....	3.472	3.677	3.572	3.808
10 per cent alcohol.....	3.864	4.666	4.536	5.600
5 per cent alcohol.....	9.184	11.172	13.160	lost
2.5 per cent alcohol....	9.968	12.306	12.432	15.120

All the specimens in 2.5 per cent alcohol were infected, so it is evident that 5 per cent alcohol is the optimum concentration to use as an antiseptic in autolysis work. It will be noticed, if the 20 per cent concentrations are compared with the controls, that this strength of alcohol does not by any means entirely stop autolysis. Tests of higher concentrations were not reported by Yoshimoto.[2]

Our experiments on this subject were performed as follows:

Experiment I. Fresh dog liver was ground fine in a hashing machine, and 5-gram samples were spread thin on pieces of filter paper to secure as nearly uniform conditions of surface as possible. These samples were then put into bottles with the amounts of alcohol indicated, tightly stoppered, and left standing at room temperature for twenty-seven days. At this time the fluid in bottles 1, 2 and 3 was distinctly more pinkish than in the higher strength of alcohol, as if the hemoglobin had been imperfectly fixed and had gone into solution. The bottles were all heated to the boiling point of their contents, to stop further autolysis, filtered, washed and the total nitrogen determined in both filtrate and insoluble residue. A duplicate set (B) was left at room temperature ninety-three days.

[1] *Zeitschr. f. physiol. Chem.*, lviii, p. 363, 1909.

[2] The action of alcohol on the antigenic properties of horse meat proteins has been investigated by Kodama (*Zeitschr. f. Hyg.*, lxxiv, p. 30, 1913) who found that the antigenic power of fresh meat was destroyed by alcohol, the time required varying directly with the strength of the alcohol.

ADDED TO TISSUE	PER CENT ALCOHOL PRESENT*	PER CENT TOTAL NITROGEN IN FILTRATE	
		A (27 days)	B (93 days)
1. 50 cc. water + toluene	0.0	59.9	57.1
2. 40 cc. water + 10 cc. 95 per cent alcohol	17.7	24.0	49.0
3. 35 cc. water + 15 cc. 95 per cent alcohol	26.4	lost	30.0
4. 30 cc. water + 20 cc. 95 per cent alcohol	35.0	20.0	23.5
5. 25 cc. water + 25 cc. 95 per cent alcohol	44.5	14.0	18.3
6. 20 cc. water + 30 cc. 95 per cent alcohol	53.0	11.0	11.0
7. 15 cc. water + 35 cc. 95 per cent alcohol	61.5	7.5	8.9
8. 10 cc. water + 40 cc. 95 per cent alcohol	70.5	8.0	8.1
9. 5 cc. water + 45 cc. 95 per cent alcohol	80.0	6.4	7.6
10. 0 cc. water + 100 cc. 95 per cent alcohol	91.3	5.7	6.1
11. 0 cc. water + 50 cc. 95 per cent boiled at once		5.2	6.2

*NOTE—In making the calculations of alcoholic strength the water contained in the tissue, amounting to about 80 per cent of normal liver, is taken into consideration. Some of the analyses were made by Helen F. Craig.

From this experiment it would seem that ground tissue undergoes measurable autolysis at room temperature when the alcohol content is between 70 and 80 per cent, but not higher. Apparently autolysis in toluene water has reached its maximum by the twenty-seventh day, but the autolysis depressed by alcohol continues more slowly, presumably to eventually reach an end-point not far from that of autolysis in toluene water. There is one possible source of error in these experiments, however, and that lies in the different alcoholic strengths of the solvent which is filtered from the residue. Conceivably some of the nitrogenous constituents of the tissues might be soluble in 70 per cent alcohol and not in 90 per cent, thus accounting for the higher nitrogen figures in the previous solution. On the other hand, an opposite effect might be produced by heating with higher strength of alcohol, through solution of lecithin. The amount of influence this factor has is shown in Experiments III and VI.

Histological examination of blocks of the same liver tissue, cut each 5 x 1 x 1 cm. and kept in the same strengths of alcohol for ninety-three days, showed that the tissues preserved in less than 45 per cent alcohol (1–4) exhibited marked vacuolization and loss of cytoplasm and an entire absence of nuclear staining. Occasionally a few stainable nuclei persist in the peripheral layers of Nos. 3 and 4. From 45 to 60 per cent alcohol preserves the tissues enough so that the nuclei may be made out, although only faintly stained, but there is still much disintegration of the cytoplasm.

With 70 per cent alcohol (No. 8) we first obtain a fair histological preparation, but when compared with the tissue preserved in 80 per cent alcohol (No. 9) it is seen to have been imperfectly preserved, for the cytoplasm is paler and more vacuolated, and many of the nuclei stain paler. Even No. 9, with an alcohol strength of 80 per cent, is distinctly inferior to No. 10 with 91.3 per cent of alcohol. These findings are in good agreement with the analytical results, showing that a definite disintegration of tissues takes place even in 80 per cent alcohol.

Experiment II. A fresh dog liver, nearly free from blood, was cut into blocks, each as nearly as possible 5 x 1 x 1 cm., which were trimmed to weigh each approximately 5 grams. This form was selected to reproduce conditions comparable to those obtaining in ordinary preparations preserved for histological work. These blocks of tissue were kept in alcohol solutions of the strengths indicated, for 190 days at room temperature, at which time the fluid was filtered off the tissue through a dry filter, and 25 cc. samples analyzed.

	Added to tissue	Per cent alcohol present	Mg. N present in filtrate per gm. tissue
1.	20 cc. water + 30 cc. absolute alcohol	55.5	2.03
2.	5 cc. water + 45 cc. 95 per cent alcohol	78.7	1.71
3.	5 cc. water + 45 cc. absolute alcohol	83.3	1.77
4.	50 cc. 95 per cent alcohol	87.9	lost
5.	50 cc. absolute alcohol	92.6	1.14
6.	100 cc. absolute alcohol	96.1	1.12
7.	50 cc. 95 per cent alcohol (boiled)		1.47

From the above experiment it would seem that even with the alcohol at 83.3 per cent strength there is a small amount of autolysis in blocks of liver tissue of the size indicated; also, that boiling in 95 per cent alcohol dissolves out some nitrogen not dissolved from tissues by cold 95 per cent or absolute alcohol.

Microscopic examinations of sections from these blocks of tissue showed in all a fairly good preservation, but in No. 1 with 55.5 per cent alcohol, the cytoplasm was a little more granular and less clearly stained, and the nuclear detail was somewhat obscured.

Experiment III. A duplicate of II, with the same liver, but with the tissue ground fine and 5-gram samples taken as in Experiment I. Here the material was extracted with the same strength solvent, by adding to all but No. 1 sufficient water to make each solution of the same strength as No. 1 (52.8 per cent) before heating to boiling temperature. The preparations were then cooled, filtered through dry filter papers, and each washed alike with 25 cc. of 50 per cent alcohol. It was noted that Nos. 1 and 2 filtered clear, but the remainder, especially No. 5 remained turbid (fats and lipoids?). The results of nitrogen determinations of the filtrates are as shown below.

	ADDED TO TISSUE	PER CENT ALCOHOL PRESENT	MG. N IN FILTRATE PER GM. TISSUE
1.	20 cc. water + 30 cc. 95 per cent alcohol	52.8	4.62
2.	5 cc. water + 45 cc. 95 per cent alcohol	78.7	2.45
3.	50 cc. 95 per cent alcohol	87.9	2.31
4.	50 cc. absolute alcohol	92.6	2.03
5.	100 cc. absolute alcohol	96.1	2.31
6.	50 cc. 95 per cent alcohol (boiled)		2.32

Here there seems to be little if any autolysis with 78.7 per cent alcohol, presumably because the ground tissue is acted upon more thoroughly by the alcohol than is the tissue in blocks.

Experiment IV. Here 10-gram samples of tissue were used, in order to reduce the source of error from determining small quantities of nitrogen. In one set the tissue was ground fine and placed on filter papers before being immersed in the alcohol; in the other the tissue was cut in blocks 1 cm. square cross section. Also, additional figures were obtained by titrating the amino-nitrogen by the formaldehyde method of Sörensen, before determining the total incoagulable nitrogen by Kjeldahl. In this series the method of collecting the soluble nitrogenous constituent was also somewhat different from that used in the preceding experiments, in that at the close of the period of autolysis the mixtures were thrown on small dry filters, and each washed with equal volumes of 95 per cent alcohol and then with water. The filtrates, which were slightly acid, were placed on the water bath until the greater part of the alcohol had been removed; they were then diluted with water, and treated with $BaCl_2$ and $Ba(OH)_2$ for the Sörensen determination. The alkaline solution was then filtered and washed, and the free ammonia and amino nitrogen determined by titration. After the titration the solutions were made distinctly acid with H_2SO_4 and again evaporated to dryness on the water bath. This residue was then taken up and the total nitrogen determined. The results obtained are given in the following table:

Added to tissue	Tissue ground—room temp. 84 days			Tissue in blocks—room temp. 79 days		
		Mg. of N. per 1 g. sample			Mg. of N. per gram tissue	
	Per cent alcohol present	NH_3 and amino acid N	Total nitrogen	Per cent alcohol present	NH_3 and amino acid N	Total nitrogen
1. 85 cc. H_2O + 15 cc. 95 per cent	13.2	11.208	17.177	13.2	13.169	19.311
2. 35 cc. H_2O + 65 cc. 95 per cent	57.2	1.110	2.707	57.2	1.215	2.498
3. 15 cc. H_2O + 85 cc. 95 per cent	74.8	0.833	2.134	74.8	0.989	2.238
4. 10 cc. H_2O + 90 cc. absolute	83.3	0.746	1.752	82.9	0.815	1.922
5. 100 cc. — 95 per cent	88.0	0.625	1.509	88.0	0.538	1.379
6. 100 cc. absolute	92.6	0.555	1.358	92.6	0.468	1.257
7. 200 cc. absolute	96.1	0.538	1.215	96.0	0.416	1.133
8. 100 cc. — 95 per cent (boiled)				87.8	0.486	1.568

From these figures it will be seen that the results by the Sörensen titration parallel very closely the soluble nitrogen determinations, showing that the increase in soluble nitrogen indicates a true proteolytic disintegration of the tissue, and that there is a certain slight autolysis at 83 per cent, with possibly a little at 88 per cent. The higher figures for No. 8 are ascribable to the boiling, which permits of more extraction from the tissues of certain nitrogenous compounds, presumably lipoids. As in previous experiments there seems to be usually a little more autolysis in the solid blocks than in the ground tissue, probably because the alcohol permeates the latter more quickly and thoroughly.

Experiment V. A duplicate of Experiment IV, except that here only the ground tissue was used, and after standing at room temperature overnight the preparations were placed in an incubator at 37°C. and kept there for 66 days. Here the results, as shown in the following table, are quite the same as to the strength of alcohol, which seems to permit a slight autolysis at 88 per cent and definite autolysis at 79 per cent. It is of interest that the amount of nitrogen set free in tissues kept in relatively high concentrations of alcohol is if anything less at incubator temperature than at room temperature, but with weak alcohol autolysis is greater at 37°C. This may be interpreted as indicating that the destruction of the enzymes by strong alcohol, and perhaps also the dehydration of the tissue proteins, is favored by the higher temperatures, but that with lower concentrations, which do not affect the enzymes so much, the usual acceleration of autolysis by temperature is exhibited.

Tissue ground—Incubator temperature (37°) 66 days.

Added to tissue	Per cent alcohol present	Mg. of N per 1 gm. sample	
		NH_3 and amino acid N	Total nitrogen
1. 85 cc. H_2O + 15 cc. 95 per cent...	13.2	15.372	22.382
2. 35 cc. H_2O + 65 cc. 95 per cent...	57.2	1.457	3.123
3. 15 cc. H_2O + 85 cc. 95 per cent...	74.8	0.746	1.770
4. 10 cc. H_2O + 90 cc. 95 per cent...	79.2	0.625	1.544
5. 100 cc. 95 per cent.....	88.0	0.486	1.353
6. 100 cc. absolute.....	92.6	0.416	1.162
7. 200 cc. absolute.....	96.1	0.434	1.110
8. 100 cc. 95 per cent (boiled).....	88.0	0.434	1.215

Experiment VI. In order to determine how much if any of the decrease in soluble nitrogen in higher concentrations of alcohol depends on simply a difference in solubility of the nitrogenous tissue constituents in various strengths of alcohol, rather than on autolytic changes, the following experiment was performed. Samples of fresh ground dog liver tissue, weighing 10 grams each, were put on filter paper and placed in alcohol of varying concentrations, a duplicate series being prepared. One set was placed on the water bath and heated near boiling for one hour; the other was not heated, and both sets were left standing at room temperature for 113 days. Nitrogen was determined in 50-cc. samples of the filtered solutions from each set, with the results given in the following table:

Nitrogen expressed in milligrams per gram of tissue.

Sample no.	Percentage concentrations of alcohol	Boiled samples, nitrogen per gm.	Unboiled, kept at room temp., 113 days, nitrogen per gm.
1.	13.2	4.594	22.798
2.	22.0	3.376	15.399
3. 30.8	30.8	2.854	9.558
4.	39.6	2.540	7.646
5.	48.4	2.332	5.664
6.	57.2	2.366	3.444
7.	66.9	2.158	2.761
8.	74.8	1.879	2.584
9.	83.6	2.158	2.195
10.	92.6	1.705	1.593

These figures indicate that alcohol of lower concentrations does dissolve somewhat more nitrogen from liver tissue than higher concentrations. The difference between strengths from 40 per

cent up to 92.6 per cent, are, however, very slight, but sufficient to be a possible source of error if not taken into account. Comparing the boiled and unboiled specimens, however, it seems evident that with the alcohol at 74.8 per cent and in all lower strengths there is a definite disintegration, but that at 83.6 per cent there seems to be none.

These figures are altogether comparable with those obtained in the previous experiments, all agreeing within reasonable limits of error. From them it may be concluded that even under the most favorable circumstances possible, with finely ground tissue spread in thin layers on filter paper and placed in large volumes of alcohol, autolysis is not certainly entirely inhibited even when the concentration of alcohol is as high as 80 per cent. Autolysis is almost certain to take place at any concentration below 80 per cent, and there may be a little disintegration of tissues even when a concentration between 80 and 90 per cent is employed. Since these figures include the water of the tissue itself, it means that in order to absolutely check autolysis not less than ten volumes of 95 per cent alcohol must be added to every gram of tissue, this giving an alcohol concentration of 88 per cent; preferably fifteen volumes should be used, and care taken to avoid the presence of any masses of tissue that are not exposed thoroughly to the alcohol. When the conditions of the experiment permit, the alcohol containing the tissue should be brought to boiling and held there for some time to certainly destroy the enzymes.

Our results make it apparent that analyses of tissues preserved in alcohol under the conditions usually prevailing, are unreliable, at least as far as indicating the presence and amounts of the nitrogenous components, and presumably the same is true of the nonnitrogenous substances to some degree. For example, the determination of the free amino acids in the human liver in acute yellow atrophy and in chloroform necrosis, reported by one of us,[3] are entirely valueless. In each of these cases the fresh liver tissue was cut into thin slices and placed in several volumes of 95 per cent alcohol, which, according to the information available at that time, should have sufficed to entirely prevent any autolytic

[3] Wells: The Chemistry of the Liver in Acute Yellow Atrophy, *Journ. of Exp. Med.*, ix, p. 627, 1907; The Chemistry of the Liver in Chloroform Necrosis (Delayed Chloroform Poisoning), this *Journal*, v, p. 129, 1908.

changes. We now know that this is not so, and we have no way of telling how much of the free amino acids found in these livers some time later was present before death, and how much came from autolysis while the tissues were preserved in the alcohol. There are many other analyses reported in the literature to which the same criticism is applicable, and it is to prevent others repeating this mistake that we make this report of our observations.

SUMMARY.

For complete suppression of autolytic disintegration of liver tissue by alcohol, the actual strength of alcohol present cannot be safely less than 90 per cent; between 80 and 90 per cent a slight autolysis may take place, and below 80 per cent alcohol concentration autolysis is certain to take place at either room or incubator temperature. For each gram of finely ground tissue at least 10 cc. and better 15 cc. of 96 per cent alcohol must be used for preservation if reliable chemical results are desired. When not contraindicated, it is best to boil the tissues a short time in the alcohol to destroy the enzymes.

THE PHOSPHORUS CONTENT OF CASEIN.

By ALFRED W. BOSWORTH and LUCIUS L. VAN SLYKE.

(From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y.)

(Received for publication, July 11, 1914.)

In a previous paper[1] from this laboratory, a method has been described for preparing casein practically ash-free, the last portion of calcium being removed by treating a solution of the casein in dilute NH_4OH with ammonium oxalate and excess of NH_4OH, and then allowing the mixture to stand about twelve hours. Casein thus prepared contains about 0.71 per cent of phosphorus. The accuracy of this figure has been questioned,[2] because it is considerably lower than that (about 0.85 per cent) hitherto commonly accepted as correct. The suggestion has been made tnat the lower figure is due to the splitting off of phosphorus from the casein molecule as the result of hydrolysis caused by prolonged contact with NH_4OH.

It is the purpose of this paper to present the results of an experimental study relating to the effects of partial hydrolysis of casein on the phosphorus content of casein preparations and also to offer an explanation as to why the higher figures that have been usually reported for the percentage of phosphorus in casein are not correct.

In connection with investigations recently carried on in this laboratory, the results of which have not been published yet, certain facts have been developed which appear to explain why the high figure usually accepted for the phosphorus content of casein is inevitably obtained in consequence of the method employed in making casein preparations. Two of the constituents of cow's milk are present in the form of colloidal solution, cal-

[1] This *Journal*, xiv, p. 203, 1913.

[2] Harden and Macallum: *Biochem. Journ.*, viii, p. 90.

cium caseinate and dicalcium phosphate. These two compounds appear to have a strong attraction for each other, as shown by the fact that, when casein is separated from milk by means of either centrifugal force or precipitation with a dilute acid, the casein always carries with it more or less dicalcium phosphate. It is evident, then, that in preparing casein by the usual method in which care is taken to avoid an excess of both acid and alkali, it is practically impossible to remove this phosphate completely. In order, therefore, to ascertain the true phosphorus content of casein, it is obviously necessary that the preparation be free from inorganic phosphorus and this can be accomplished only by removing all of the calcium. Several methods have been tried in this laboratory to effect this, and the one finally found to be the most satisfactory is that described in a previous paper, referred to above.

Further, a good reason for believing that the lower figure more closely approximates the truth than the higher one hitherto commonly accepted as correct is the relation of phosphorus to the molecular weight of casein. In a previous paper[3] it was shown that the molecular weight of casein is approximately 8888. Now, if the casein molecule contains two atoms of phosphorus, the percentage of phosphorus is 0.698, while the phosphorus content would be 1.046 per cent if there were three atoms of phosphorus. The figure (0.85 per cent) heretofore regarded as correct represents, therefore, on account of the presence of impurities in the preparation, neither two atoms nor three atoms of phosphorus, while the lower figure (0.71 per cent) represents almost exactly two atoms.

Coming now to the criticism made that an excess of NH_4OH in contact with casein for twelve hours causes hydrolysis, resulting in the formation of inorganic phosphorus, there is reason to believe that, whatever hydrolysis takes place, it does not necessarily interfere with the composition of the final preparation, because as will be shown, the products of hydrolysis are not precipitated by dilute acetic acid and therefore form no part of the completed preparation, which is pure, unhydrolyzed casein.

[3] This *Journal*, xiv, p. 228, 1913.

EXPERIMENTAL.

After giving the ash and phosphorus content of several preparations of casein, we will present the results of a study of two special preparations of casein which were subjected to varying conditions in order to ascertain whether hydrolysis affects the phosphorus content of casein preparations.

Ash content and phosphorus content of casein. The percentage of ash and phosphorus in five samples of casein prepared in this laboratory during the past seven or eight years is as follows:

SAMPLE	ASH PER CENT	PHOSPHORUS PER CENT
1	0.06	0.710
2	0.39	0.732
3	0.61	0.830
4	0.61	0.839
5	3.93	0.941

The results show that increase of ash is accompanied by an increase of phosphorus.

Phosphorus content of casein preparations treated in different ways. In order to study the effect of treating casein in different ways upon the content of phosphorus, and expecially to ascertain what effect partial hydrolysis may have upon the phosphorus content of casein preparations, two preparations of casein were made and each of these was treated in the manner described below.

Preparation A was made in the usual way, treating alternately with dilute acetic acid and ammonia, avoiding an excess of each reagent. This preparation contained 0.857 per cent of phosphorus.

Preparation B was made according to the method given in a previous paper,[4] the distinctive feature of which is treatment of a solution of casein in dilute alkali with ammonium oxalate and excess of alkali. This preparation contained 0.711 per cent of phosphorus.

(1) Treatment with excess of ammonia. Each of preparations A and B (20 grams) was dissolved in dilute NH_4OH and an excess

[4] This *Journal*, xiv, p. 203, 1913.

of the same reagent was added; after standing twelve hours at 37°C., the solution was centrifugalized and filtered, the casein in the filtrate being then precipitated with dilute acetic acid. This precipitated casein was washed, redissolved, reprecipitated and finally washed with water, alcohol and ether.

In the case of preparation A, the yield was 14 grams, containing 0.841 per cent of phosphorus; in the case of preparation B, the yield was 15 grams and the phosphorus content 0.713 per cent.

The decreased yield in each case was due in part to hydrolysis of casein and in part to mechanical losses. It is evident that partial hydrolysis of casein preparations has no effect on the percentage of phosphorus in the unhydrolyzed casein that is recovered.

(2) Treatment with ammonium oxalate and excess of ammonia. Each of preparations A and B (20 grams) was dissolved in dilute NH_4OH and then ammonium oxalate and an excess of NH_4OH added, the mixture being allowed to stand twelve hours at 37°C. The casein was separated as before.

In the case of preparation A, the yield was 14 grams, containing 0.723 per cent of phosphorus; in the case of preparation B, the yield was 14.5 grams, containing 0.71 per cent of phosphorus.

In these two experiments, hydrolysis of casein by alkali has no effect upon the percentage of phosphorus in the casein finally recovered. In the case of preparation A, the phosphorus content is reduced from 0.857 to 0.723 per cent, as a result of the removal of calcium phosphate from the casein preparation. In the case of preparation B, the phosphorus content remains the same as in the original preparation, because the casein used had already been subjected to treatment with ammonium oxalate and excess of NH_4OH, the calcium phosphate having been removed as completely as practicable.

(3) Treatment as in (2) but prolonged. Preparation B (20 grams) was treated as in the preceding experiment, except that the mixture was allowed to stand seventy-two hours (instead of twelve) at 37°C. The amount of casein recovered was 12.4 grams containing 0.721 per cent of phosphorus. The prolonged treatment giving opportunity for increased hydrolysis of casein, did not change the percentage of phosphorus in the casein recovered.

SUMMARY.

The amount of phosphorus in casein has been commonly given as about 0.85 per cent. By treating a solution of casein in dilute NH_4OH with ammonium oxalate and an excess of NH_4OH and letting stand 12 hours the phosphorus content is reduced to about 0.70 per cent. This lower percentage can not be explained as being due to hydrolysis of casein and splitting off of phosphorus. While some of the casein is hydrolyzed, this portion does not enter into the final preparation and does not affect its composition, because the hydrolyzed portion is not precipitated by acetic acid while the unhydrolyzed part is. The higher figure ordinarily given is due to the presence of inorganic phosphorus (dicalcium phosphate) carried from the milk into the precipitated casein and not entirely removed under the usual conditions of preparation. The lower figure corresponds very closely to two atoms of phosphorus (0.698 per cent) in the casein molecule. Analyses of various preparations of casein containing varying amounts of ash show a general correspondence between the ash and phosphorus content.

THE CAUSE OF ACIDITY OF FRESH MILK OF COWS AND A METHOD FOR THE DETERMINATION OF ACIDITY.

By LUCIUS L. VAN SLYKE and ALFRED W. BOSWORTH.

(From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y.)

(Received for publication, July 11, 1914.)

The usual method employed in determining the acidity of milk is to add a few drops of a solution of phenolphthalein as indicator to 100 cc. of milk and then titrate with $\frac{N}{10}$ NaOH. By the use of this method it is found that 100 cc. of milk, when strictly fresh, will require the addition of 15 to 20 cc. of the alkali in order to produce a faint but permanent pink coloration.

The acidity of fresh milk has been commonly attributed to the presence of acid phosphates and casein, and we will now consider the relation of these constituents to milk acidity.

That the acidity of milk is due to the presence of acid phosphates (M H_2PO_4) is indicated by the fact that milk is strongly alkaline to methyl orange. Further, it is well known that phosphates can not be titrated with any degree of accuracy in the presence of calcium salts, due to the fact that some of the insoluble dicalcium phosphate ($CaHPO_4$), which is formed during the titration, hydrolyzes, changing into calcium hydroxide and phosphoric acid, and then the calcium hydroxide unites with more dicalcium phosphate, forming tricalcium phosphate ($Ca_3P_2O_8$).[1] These facts may be represented by the following equations:

$$(1)\quad CaHPO_4 + 2\,H_2O \rightleftarrows Ca(OH)_2 + H_3PO_4$$
$$(2)\quad 2\,CaHPO_4 + Ca(OH)_2 \longrightarrow Ca_3P_2O_8 + 2\,H_2O.$$

That tricalcium phosphate is formed during the titration of any solution containing phosphoric acid and calcium salts is easily demonstrated by an analysis of the precipitate always appearing; this precipitate is tricalcium phosphate, which is characterized by its appearance, varying from a flocculent to a gelatinous con-

[1] Cameron and Hurst: *Journ. Amer. Chem. Soc.*, xxvi, p. 905, 1904.

dition according to the concentration of the calcium and phosphates in the solution.

Dibasic phosphates are neutral to phenolphthalein and monophosphates are acid to this indicator; phosphoric acid, therefore, acts as a diabasic acid to phenolphthalein. In the reaction represented above, we have, in place of the original molecule of neutral dicalcium phosphate, one molecule of free phosphoric acid, whereby the acidity as measured by titration is increased over what it would be if no such reaction occurred. These facts serve to explain some results obtained by us in connection with the study of certain problems relating to milk.

We have found that when we titrate whole milk with alkali, in the usual way and then similarly titrate the serum obtained by filtering the milk through a porous procelain filter, the titration figure given by the whole milk is about double that obtained with the serum. For example, 100 cc. of whole milk may show an acidity of 17 cc. of $\frac{N}{10}$ alkali, and 100 cc. of serum, 8 cc. This difference has ordinarily been interpreted as being due to the acidity of milk-casein, but in a future paper we shall show that casein is present in fresh milk as a calcium caseinate that is neutral to phenolphthalein. The other constituents removed from the milk by filtering through porous porcelain are fat and dicalcium phosphate, both of which also are neutral to phenolphthalein. From the illustration given above, the titration figure of the residue on the filter would appear to be 9 (17–8) for 100 cc. of milk, though in reality the reaction is neutral. We believe that the cause of this discrepancy is to be found in the dicalcium phosphate which is present in the whole milk but which is not present in the serum. Its presence in the milk permits the formation of relatively large amounts of phosphoric acid and tricalcium phosphate, requiring the use of increased amounts of $\frac{N}{10}$ alkali (17 cc.) to neutralize the milk, as compared with the amount (8 cc.) needed to neutralize the serum. We have been led by such results to believe that the acidity of milk, as usually determined, is about twice what it should be.

The disturbing influence of calcium salts in the presence of phosphates has been studied by Folin[2] in connection with the

[2] *Amer. Journ. of Physiol.*, ix, p. 265, 1903.

determination of acidity in urine; he was able largely to overcome the difficulty by the addition of neutral potassium oxalate, by which the calcium is removed in the form of the insoluble oxalate. He showed that by this preliminary treatment, correct titration figures could be obtained for monocalcium phosphate, which without such treatment gives figures that are remote from the calculated acidity.

Making use of Folin's procedure, and, before titrating with alkali, adding to milk some saturated solution of neutral potassium oxalate, we are able to obtain figures which conform more closely to the results indicated as accurate by other considerations.

The method, as modified by us for the determination of acidity in milk, whether fresh or otherwise, is as follows:

Measure 100 cc. of milk into a 200 cc. Erlenmeyer flask, add 50 cc. of distilled water and 2 cc. of a saturated solution of neutral potassium oxalate, allow the mixture to stand not less than two minutes and then titrate with $\frac{N}{10}$ NaOH. Since most solid potassium oxalate is acid, care must be taken to prepare a solution that is really neutral, which may be done in the following way: A saturated solution of ordinary potassium oxalate is prepared and decanted from the solid residue. To this solution is added 1 cc. of phenolphthalein solution and then, drop by drop, enough normal NaOH solution to produce a permanent faintly pink coloration.

In the following table is given the acidity of 21 samples of milk from individual cows, as determined by the two methods, with and without addition of neutral potassium oxalate.

SUMMARY.

The acidity of fresh milk is due to the presence of acid phosphates. Titration of phosphoric acid with alkali, in the presence of calcium salts, results in hydrolysis of dicalcium phosphate formed during the titration, whereby free calcium hydroxide and phosphoric acid are first formed and then calcium hydroxide unites with more dicalcium phosphate to form insoluble tricalcium phosphate. As a result of these reactions more alkali is required to make a solution, containing calcium and phosphoric acid, neutral to phenolphthalein than is required in the

absence of calcium. The calcium must be removed previous to titration by treatment of 100 cc. of milk with 2 cc. of saturated solution of neutral potassium oxalate.

NUMBER OF SAMPLES	AMOUNT OF $\frac{N}{10}$ NaOH REQUIRED TO NEUTRALIZE 100 CC. OF MILK	
	Before addition of neutral potassium oxalate	After addition of neutral potassium oxalate
	cc.	cc.
1	15	6.4
2	15.2	7.0
3	15.6	6.8
4	16.0	6.8
5	17.0	8.0
6	17.0	8.0
7	17.2	8.0
8	17.6	9.0
9	17.8	8.8
10	18.0	9.0
11	18.2	9.6
12	18.4	9.6
13	18.4	9.4
14	18.6	9.4
15	18.6	9.4
16	19.0	9.4
17	19.2	10.0
18	19.4	10.4
19	20.0	9.8
20	22.0	12.8
21	23.8	14.0

NOTE ON THE PROTEINS OF THE BLOOD OF *LIMULUS POLYPHEMUS* L.

By CARL L. ALSBERG.

(*From the Laboratory of the U. S. Bureau of Fisheries at Woods Hole, Mass.*)

(Received for publication, July 13, 1914.)

The blood of *Limulus polyphemus* L. has been shown to contain the protein haemocyanin and white corpuscles. The blood clots with great rapidity. Loeb[1] found that clotting is not preventable by the usual chemical methods but only by methods which prevent agglutination of the corpuscles. Further, Loeb has shown that the clot is formed by the agglutination of the corpuscles, indicating the absence of fibrinogen in the blood. By a study of the composition of the clot Alsberg and Clark[2] showed that the clot contains no fibrin, but is composed mainly of a protein, the analysis of which showed C : S : N ratios more like those of glutins than fibrins. The nitrogen content of the clot protein was found to be 15.6 per cent. Haemocyanin, which remains in the serum, contains 16.8 per cent of nitrogen. This serum has a specific gravity of 1.040 as found by Gotch and Laws.[3] They first studied the haemocyanin of Limulus and concluded that heating the serum does not completely coagulate the haemocyanin, because the liquid still remains blue after boiling. Howell[4] states that in order to produce complete coagulation it is necessary to heat the

[1] Loeb, L.: Untersuchungen über Blutgerinnung, *Beitr. z. chem. Physiol. u. Path.*, vi, p. 279, 1905.

[2] Alsberg, C. L. and E. D. Clark: The Blood Clot of *Limulus polyphemus*, this *Journal*, v, p. 323, 1908.

[3] Gotch, F. and J. P. Laws: On the Blood of *Limulus polyphemus*, Report of 54th meeting of the British Association for the Advancement of Science, 1884, pp. 774–6, London, 1885.

[4] Howell, W. J.: Observations on the Chemical Composition of the Blood of *Limulus polyphemus*, *Johns Hopkins University Circular*, v, pp. 300–335, 1885.

serum a long time at 80°. Halliburton[5] gives the coagulation temperature at 65-66°. Alsberg and Clark[6] prepared the haemocyanin of Limulus by fractional precipitation with ammonium sulphate and removal by dialysis of the ammonium sulphate from the purified product. The substance purified in this way could not be crystallized. It differs from the haemocyanin of Octopus investigated by Henze,[7] both in its percentage composition and in its physical characteristics. Limulus haemocyanin is easily precipitated, practically quantitatively, by weak acids, but by this method of precipitation more or less of the copper is dissociated. It is, also, precipitated, practically quantitatively, by dilute solutions of zinc sulphate. This method was first employed by Kobert[8] to precipitate the haemocyanin of *Eledone moschata*. Fredericq[9] has shown that haemocyanin is the only protein in the plasma of Octopus. Henze confirmed the results of Fredericq and found, further, that haemocyanin constituted 9 per cent of the blood. From the observations of Fredericq,[10] who found that the protein content of the blood of the river crayfish, *Astacus fluviatilis*, varies according to the state of nutrition of the animal, it would seem that the protein content of the blood of Crustacea is quite variable. Slight variability is well known to occur in higher animals including man, as shown by Voit,[11] Lewinski[12] and Benedict.[13]

In view of the scanty data existing concerning the distribution of nitrogenous substances in the blood of the lower animals, further information on this subject would seem to be desirable.

[5] Halliburton: On the Blood of *Decapod crustacea*, *Journ. of Physiol.*, vi, pp. 300-335, 1885.

[6] Alsberg, C. L. and E. D. Clark: The Haemocyanin of *Limulus polyphemus*, this *Journal*, viii, p. 1, 1910.

[7] Henze, M.: *Zeitschr. f. physiol. Chem.*, xxxiii, pp. 370-389, 1901.

[8] Kobert, R.: Ueber Haemocyanin nebst einigen Notizen über Haemerythrin, *Pflüger's Archiv*, xcviii, p. 411, 1903.

[9] Fredericq, L.: Influence du milieu ambiant sur la composition du sang des animaux aquatiques, *Arch. de Zool.*, 2 Série, iii, 1885.

[10] Fredericq, L.: Note sur le sang de l'ecrivisse, *Libre jubil. dedié à Charles Van Bambeke*, Bruxelle, 1899.

[11] Voit, E.: *Zeitschr. f. Biol.*, xlvi, p. 195, 1904.

[12] Lewinski, J.: *Pflüger's Archiv*, c, p. 611, 1903.

[13] Benedict, F. G.: Publication No. 77, Carnegie Institution of Washington.

Some information concerning the blood of *Limulus polyphemus* is given in the following work. At the same time, since the observations on the coagulation of haemocyanin differ, the behavior of this substance to heat was studied.

For the observations on the distribution of nitrogen in the blood and in the serum of Limulus, blood from animals which had been kept from one to twelve weeks in a float in the harbor at Woods Hole was drawn directly into a tared bottle and weighed. In Table I the order of experiments indicates decreasing length of time after capture. In each of the first four experiments of Table I blood was taken from a different animal. In the fifth experiment a measured volume of blood was taken from a number of animals. Its weight was estimated by calculating the weight of

TABLE I.

Distribution of Nitrogen in Limulus Blood.

NO.	BLOOD	N IN CLOT	PROTEIN IN CLOT		N IN HAEMOCYANIN	HAEMOCYANIN	
	grams	*gram*	*grams*	*percent of blood*	*gram*	*gram*	*percent of blood*
1	18.26	0.0045	0.0288	0.158	0.0483	0.298	1 63
2	26.004	0.0104	0.0672	0.258			
3	28.393	0.0135	0.0865	0.304			
4	53.424	0.0494	0.316	0.590			
5	393.5*	0.376	2.403	0.610			

* This determination was made on 390 cc. of composite blood of a number of animals.

the measured expressed serum, assuming that 1.040 was its specific gravity. Obviously the figure obtained is not strictly comparable with the others given in the table, because the influence of the clot protein is not considered. During the clotting, as shown in the table, the blood yielded 390 cc. of serum and 2.4 grams of clot protein. In all experiments the weighed portions of blood were kept at about 10°C. in an ice box for twenty-four hours. The clot, after draining carefully from supernatant liquid, was washed first with 5 per cent sodium chloride solution, then with distilled water. Nitrogen in the insoluble residue was then determined by the Kjeldahl method. In determination 1 of Table I the haemocyanin was completely precipitated in the filtrate from the clot by 10–15 cc. of 5 per cent zinc sulphate solution. The

precipitated haemocyanin, separated by filtration, was washed with 5 per cent zinc sulphate solution. Nitrogen in the residue was determined. After saturating the filtrate from the haemocyanin with zinc sulphate, an insignificant precipitate was formed, which contained only traces of nitrogen. The calculations of clot protein and of haemocyanin in Table I and of haemocyanin in Table II are based on the nitrogen content for the respective proteins as given above.

It was found that the filtration of solutions containing zinc sulphate was too slow to allow suitable washing of residues. Therefore, in all subsequent experiments recorded in Table II the haemocyanin was precipitated by dilute acetic acid. The precipitate was removed by filtration through paper, washed with

TABLE II.

Nitrogen Distribution in Limulus Serum.

No.	Serum	N in haemocyanin	Haemocyanin		N in coagulum after removal of haemocyanin	Non-coagulable N
	cc.	*gram*	*gram*	*percent of serum*	*gram*	*gram*
1	25*	0.1204	0.744	2.94	0.0011	0.0073
	25*	0.1210	0.747	2.96	0.0012	0.0072
2	25	0.0755	0.466	1.84	0.0020	0.0115
3	25	0.0696	0.430	1.74	0.0014	0.0112

* These are duplicates.

water faintly acidulated with acetic acid and its nitrogen content determined. The filtrate was nearly neutralized with sodium carbonate and brought to boiling. The coagulum was removed by filtration on paper, washed and its nitrogen content determined. Nitrogen was also determined by the Kjeldahl method in the filtrate.

One fact clearly brought out by a study of these tables is that in the blood of *Limulus polyphemus* very little protein other than the clot protein and the haemocyanin is present. For, after the removal of both of these proteins saturation with zinc sulphate indicates the presence of a minute amount of nitrogen in non-coagulable form. Its properties were not studied. Since the clot of Limulus blood is formed by the agglutination of the cells,

as shown by Loeb,[14] a considerable amount of cell protein might be expected to pass into the blood. Apparently, this cell protein is insignificant in amount, the greater part of the proteins of the blood cells of Limulus remaining in the clot. However, a minute amount of other proteins does occur, as shown by the very slight precipitate formed on saturating with zinc sulphate, or, of coagulum formed by heating the serum deprived of clot protein and haemocyanin.

There seems also to be present in the clot a minute amount of protein which can be crystallized in macroscopic rhombic plates the largest of which measure one-eighth of an inch. The yield was so small that no further study was attempted.

Another fact shown by the tables is that the amount of haemocyanin present in the blood is much less than is found in the blood of Octopus, estimated by Henze as 9 per cent.

Inspection of the tables shows, moreover, that the clot protein and the haemocyanin vary greatly in amount in different individuals. In spite of these variations it is clear that the haemocyanin is several times as abundant as the clot protein. The variations themselves are probably dependent upon the condition of nutrition of the animal. Since, as stated above, the specimens examined for the results recorded here had been kept in a float in the harbor of Woods Hole for varying periods, some of them were in a more or less advanced stage of starvation. After three months of confinement the protein content of the blood diminished from approximately 3.5 per cent to 1.50 per cent. Similar observations on the influence of starvation on the protein content of the blood of other animals are cited above.

For the determination of the coagulation temperature pure neutral solutions of haemocyanin containing a little ammonium sulphate were heated. These were obtained after precipitation of serum with the requisite amount of ammonium sulphate,[15] the precipitate filtered off, redissolved and dialyzed. When heated to 48°C. opalescence appeared. This became more marked as the temperature rose but distinct floccules did not appear until 60–62°C., while the coagulation did not seem to be complete until

[14] Loeb, L.: *loc. cit.*

[15] Alsberg, C. L. and E. D. Clark: *loc. cit.*

67–68°C. was reached, which accords very well with Halliburton's determination of 65–66°C. This is the behavior of solutions of pure haemocyanin; serum does not coagulate so readily. The observations of Howell, and of Gotch and Laws, that the haemocyanin in serum does not readily coagulate completely are in accord with the observations recorded here. The different behavior of haemocyanin in serum and in pure solution is due to the alkalinity of the serum. That alkalinity greatly affects the coagulability of protein is well known.[16] The failure to take this alkalinity into consideration seems to have led to a certain amount of confusion in the observations of those investigating invertebrate bloods.

The small amount of protein obtained by coagulation after removal of the clot and haemocyanin, as recorded in Table II, seems to be different from the haemocyanin, as far as may be judged from the coagulation point. It begins to coagulate at 66°C., but coagulation is slight until a temperature of 72–74°C. is attained. Evidence such as this based upon the temperature of coagulation can not, of course, be regarded as conclusive.

SUMMARY.

The proteins of the blood of Limulus consist almost exclusively of the clot protein, or cell fibrin, and haemocyanin. Haemocyanin is several times as abundant as the cell fibrin. Other proteins occur only in minimal amounts. The blood also contains a small amount of nitrogen in non-coagulable form. The quantity of protein in the blood seems to vary with the condition of the animal, diminishing in starvation. There is probably, at most, less than half as much haemocyanin as in the blood of Octopus. The coagulation temperature of Limulus haemocyanin was found to be 67–68°C.

[16] Chick, H. and C. J. Martin: On the "Heat Coagulation" of Proteins. Part III. The Influence of Alkali upon the Reaction Velocity, *Journ. of Physiol.*, xlv, p. 61, 1912.

THE METABOLISM OF AN ISOMER OF XANTHINE AND SOME ISOMERS OF THE METHYLXANTHINES.[1]

By SAMUEL GOLDSCHMIDT.

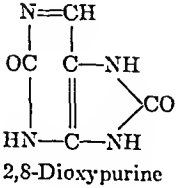
(From the Sheffield Laboratory of Physiological Chemistry, Yale University New Haven, Connecticut.)

(Received for publication, July 17, 1914.)

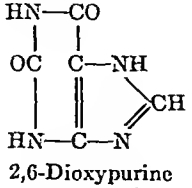
The investigation of the metabolism of purines in recent years has furnished a fairly clear understanding of the interrelations of certain well known members of this group of compounds. The current ideas regarding the origin of uric acid or allantoin for example, through successive well defined stages from adenine and guanine to hypoxanthine, xanthine, etc., are based upon extensive experimental evidence which need not be reviewed here. In the last few years a considerable number of new purine derivatives has been prepared synthetically. The problems of the physiological behavior of purine compounds isomeric with those more commonly found in nature has scarcely been subjected to investigation. In view of the marked specificity exhibited by the purine-converting enzymes already known, the fate of the newer purine compounds, especially those closely related to the purine representatives occurring in biological tissues and fluids deserves careful study.

RELATION OF THE SYNTHETIC PURINES STUDIED TO THE NATURALLY OCCURRING PURINES AND TO SOME OF THE PURINES APPEARING IN THE URINE UNDER SPECIAL CONDITIONS.

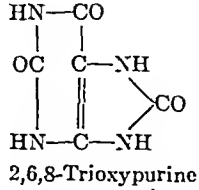
2,8-Dioxypurine.



2,8-Dioxypurine



2,6-Dioxypurine (Xanthine)

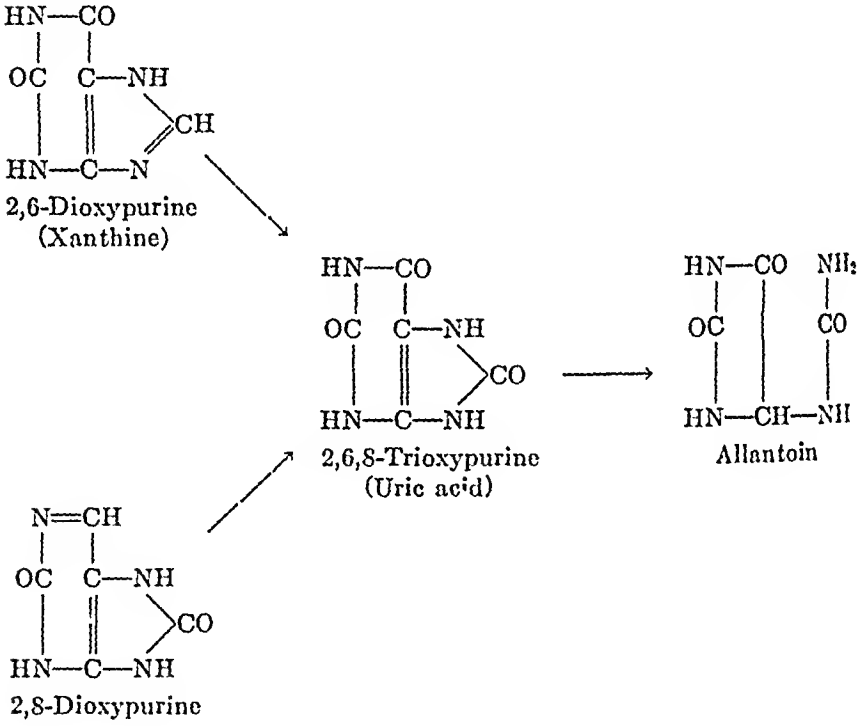


2,6,8-Trioxypurine (Uric acid)

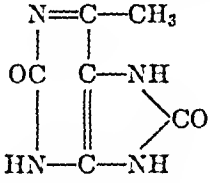
[1] The experimental data in this paper are taken from the dissertation submitted by the writer for the degree of Doctor of Philosophy, Yale University, 1914.

2,8-Dioxypurine (Johns, 1910) is an isomer of xanthine and a homologue of uric acid. One might with reason suppose that this purine would undergo the same oxidation in the animal organism as does xanthine. In rabbits this would mean conversion either to uric acid or allantoin. If we assume, as is currently believed, that xanthine is first converted to uric acid and then to allantoin in the animal organism, it would be reasonable to expect the oxidation in the case of 2,8-dioxypurine to take place in the 6 position as readily as the familiar 2,6-dioxypurine is oxidized in the 8 position.

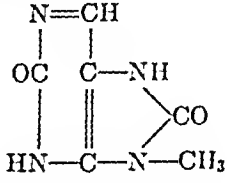
This possibility is indicated in the following scheme:



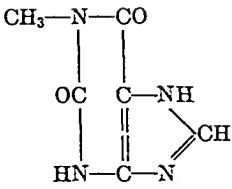
Monomethylpurines.

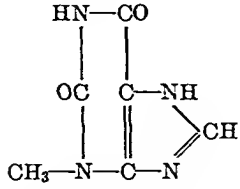


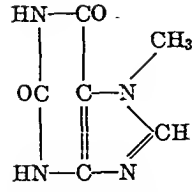
2,8-Dioxy-6-methylpurine



2,8-Dioxy-9-methylpurine

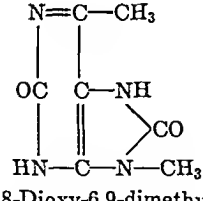

1-Methylxanthine

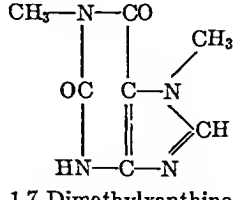

3-Methylxanthine

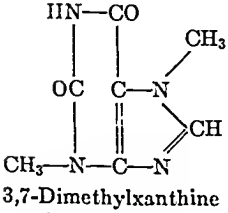

7-Methylxanthine

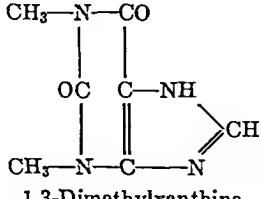
2,8-Dioxy-6-methylpurine (Johns, 1909) and 2,8-dioxy-9-methylpurine (Johns, 1911) are isomers of the monomethylxanthines. If, as most observers believe, the monomethylxanthines are excreted unchanged when introduced into the body, we would expect these synthetic monomethylpurines to act likewise. On the other hand, if the monomethylxanthines may be demethylated, as the work of Schittenhelm (1910) indicates, these synthetic purines might be converted to uric acid, or to allantoin for the most part, in the rabbit. Furthermore, the possibility of a diuretic action suggests itself for these compounds. Of the monomethylxanthines, 3-methylxanthine has been found to exhibit this effect. 7-Methylxanthine exerts this property to a slight extent only (Ach, 1900; Albanese, 1900).

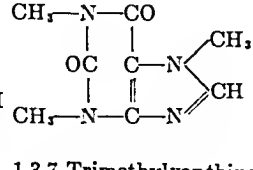
Dimethylpurines.


2,8-Dioxy-6,9-dimethylpurine


1,7-Dimethylxanthine (Paraxanthine)


3,7-Dimethylxanthine (Theobromine)


1,3-Dimethylxanthine (Theophylline)


1,3,7-Trimethylxanthine (Caffeine)

2,8-Dioxy-6,9-dimethylpurine (Johns, 1912) is an isomer of theobromine, theophylline and paraxanthine, and a homologue of caffeine. Like the dimethylxanthines, this purine presents two possibilities of metabolism; either a partial, or a complete demethylation with oxidation to uric acid or allantoin in the rabbit. It differs from the naturally occuring dimethylpurines in having one of its methyl groups attached to a carbon atom instead of to a nitrogen. With this compound a diuretic action might be looked for. This property is exhibited by all of the dimethylxanthines and by caffeine; in fact, theobromine (3,7-dimethylxanthine) exhibits the strongest diuretic effect hitherto obtained from purines (von Schröder, 1887; Ach, 1900).

METHODS.

The injections were all made subcutaneously into rabbits in solutions of 10 to 15 cc. volume distributed over various parts of the abdomen; the total volume never exceeded 50 cc. Unless otherwise designated, the purines were dissolved in the least possible amount of sodium hydroxide solution; an excess was required to maintain a perfect solution. This excess of sodium hydroxide usually caused a necrosis at the site of injection; but as a rule, this did not occur until after the end of the experiments. In a few of the experiments lithium carbonate solution was used as the solvent, with the result that no necrosis took place.

The urine was collected in daily periods by applying pressure on the bladder through the abdominal wall. Each day's urine was made up to a definite volume and preserved with toluene until the end of the experiment. Total nitrogen was determined by the Kjeldahl method. Aliquot portions of each day's urine were taken for the allantoin and purine base determinations for each period. In the experiment on rabbit 4 allantoin was determined by the original Wiechowski method;[2] the substance was crystallized and weighed. In the remainder of the experiments the modified Wiechowski method[3] was employed; nitrogen was determined in the mercuric acetate precipitate. Uric acid

[2] Wiechowski: *Beitr. z. chem. Physiol. u. Path.*, xi, p. 109, 1908.
[3] Wiechowski: *Neubauer-Huppert, Analyse des Harns*, ii, p. 1076, 1913.

and purine bases other than uric acid were determined by the Krüger-Schmid method,[4] as outlined in Neubauer-Huppert.[5]

In experiments designed to test the accuracy of the modified Wiechowski method, the writer was able to recover only 74 per cent of the pure allantoin dissolved in rabbit's urine. Daily determinations on the urine gave results which agreed very closely when the rabbits were given the same diet, so that, for the purpose of these experiments, the comparative value of the results are not affected.

The synthetic purines were made under the direction of Prof. C. O. Johns, and were carefully purified. The specimen of xanthine used was a pure synethetic product made by Prof. C. O. Johns. The uric acid was a Kahlbaum preparation, repurified.

EXPERIMENTS.

2,8-Dioxypurine.

Rabbits 1 and 2 were used to test a possible toxicity or diuretic effect of this compound. The urine was collected in two periods each day, so that any prompt increase in urinary volume on the experimental day above the normal output could be readily detected. As a control, the animals were injected during a fore period with an amount of physiological salt solution equivalent to the volume to be injected later. In neither case was any diuretic effect observed. The animals showed no toxic symptoms as a result of the injection.

Rabbits 4 and 5, after receiving injections of this purine, failed to show any increase in their allantoin output. The increase in total nitrogen output on each experimental day accounts for the purinc nitrogen injected.

In rabbit 25 the purine was injected in a solution of lithium carbonate. Here, also, no increase of the allantoin output occurred; in fact, the excretion of allantoin in the experimental period was less than that in the fore period. The output of nitrogen on the day of the injection accounts for the injected nitrogen. In this experiment the periods were increased to three

[4] Krüger and Schmid: *Zeitschr. f. physiol. Chem.*, xlv, p. 1, 1905.
[5] Neubauer-Huppert: *Analyse des Harns*, ii, pp. 946, and 983, 1913.

days each in order to take into account any possibility of tardy absorption of the purine in the experimental period.

Isolation of 2,8-dioxypurine from the urine. 0.45 gram of 2,8-dioxypurine was injected into a rabbit. After an interval of two days during which time the urine was collected, another injection of 0.55 gram was made. The urine was collected for three days after the last injection.

The total quantity of urine obtained during the experiment was evaporated to a small volume and acidifed with acetic acid. A precipitate formed which was purified several times by dissolving it in sodium hydroxide and reprecipitating with acetic acid.

Weight of the crude precipitate	0.70 gram
Weight of purine injected	0.99 gram

Finally the precipitate was dissolved in 5 cc. of normal sodium hydroxide with the result that crystals of a sodium salt were obtained.

The sodium salt of 2,8-dioxypurine contains four molecules of water of crystallization. The sodium salt of xanthine contains only one molecule of water of crystallization (Johns, 1910).

	Calculated for: $Na.C_5H_3O_2N_4.4H_2O$	Found:
H_2O	29.26	27.91
	$Na.C_5H_3O_2N_4$	Found:
N	32.18	32.08

0.050 gram of the sodium salt was dissolved in water and the solution acidified with acetic acid. The free purine was dried and gave the following analysis:

	Calculated for: $C_5H_4O_2N_4$	Found:
N	36.84	35.99

Rabbit 1. 2,8-Dioxypurine.

DAY	WEIGHT	URINE					DIET PER DAY: 150 gms. carrots 20 gms. oats
		Volume 9 a.m. –5 p.m.	Volume 5 p.m. –9 a.m.	Total volume 24 hours	Specific gravity 24 hours	Total N	
	kilos	*cc.*	*cc.*	*cc.*		*gram*	
1	1.44	24	62	86	1.030	0.67	
2		61	60	121	1.034	0.47	
							3d day:
3		61	39	100	1.023	0.37	Injected 25 cc. of physiological saline at 9.15 a.m.
4		54	52	106	1.040	0.37	
							5th day:
5	1.38	60	54	114	1.020	0.33	Injected 0.2576 gm. of 2,8-dioxypurine at 9.15 a.m.= 0.094 gm. N.

Rabbit 2. 2,8-Dioxypurine.

DAY	WEIGHT	URINE					DIET PER DAY 225 gms. carrots 20 gms. oats
		Volume 9 a.m. –5 p.m.	Volume 5 p.m. –9 a.m.	Total volume 24 hours	Specific gravity 24 hours	Total N	
	kilos	*cc.*	*cc.*	*cc.*		*gram*	
1	2.52	61	72	133	1.030	0.66	
2		100	73	173	1.015	0.53	
							3d day:
3		75	86	161	1.030	0.41	Injected 25 c.c. of physiological saline at 9.15 a.m.
4		92	62	154	1.020	0.36	
							5th day:
5		112	90	202		0.60	Injected 0.2461 gm. 2,8-dioxypurine at 9.15 a.m.= 0.0899 gm. N
6	2.48			130	1.030	0.46	

Rabbit 4. 2,8-Dioxypurine.

DAY	WEIGHT	URINE				DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Allantoin per day	
	kilos	*cc.*		*gram*	*gram*	
1	2.5	174	1.020	0.44	0.0198	
2	2.5	160	1.020	0.47		
3	2.5	166	1.028	0.67	0.0266	3d day: Injected 0.3042 gm. 2,8-dioxypurine = 0.111 gm. N.
4	2.5	170	1.028	0.47	0.028	
5	2.5	150	1.022	0.44		

Rabbit 5. 2,8-Dioxypurine.

DAY	WEIGHT	URINE				DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Allantoin per period	
	kilos	*cc.*		*gram*	*gram*	
1	2.44	178	1.020	0.69	0.127	
2	2.46	172	1.020	0.66		
3	2.40	218	1.014	0.82	0.1068	3d day: Injected 0.5093 gm. of 2,8-dioxypurine = 0.186 gm. N.
4	2.36	180	1.020	0.67		
5	2.34	164	1.026	0.57	0.130	
6	2.34	142	1.024	0.47		

Rabbit 25. 2,8-Dioxypurine.

DAY	WEIGHT	URINE				DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Allantoin per period	
	kilos	*cc.*		*gram*	*gram*	
1	1.90	192	1.020	0.99	0.202	
2	1.86	150	1.026	0.83		
3	1.86	160	1.024	0.70		
4	1.82	140	1.024	0.86	0.177	4th day: Injected 0.3424 gm. 2,8-dioxypurine as a lithium salt = 0.125 gm. N.
5	1.80	150	1.028	0.77		
6	1.82	200	1.020	0.72		
7	1.76	170	1.018	0.84	0.171	
8	1.74	140	1.026	0.68		
9	1.74	160	1.030	0.74		

2,8-Dioxy-6-methylpurine.

This purine, when injected into rabbit 7, produced no apparent toxic symptoms or diuresis.

Rabbit 8, after being injected with this compound, excreted no increase of allantoin over the fore period. In fact, the output of allantoin during the experimental period was less than during the fore period but agreed very closely with that of the after period.

Isolation of 2,8-dioxy-6-methylpurine from the urine. 0.60 gram of 2,8-dioxy-6-methylpurine was injected into a rabbit and after an interval of two days another injection of 0.58 gram was made. The urine was collected throughout the whole time and for three days after the last injection. The urine collected was subjected to the same treatment as described above for the isolation of 2,8-dioxypurine. The purified product weighed 0.12 gram, 10 per cent of the total amount of purine injected, and gave the following analysis:

	Calculated for: $C_6H_6O_2N_4$	Found:
N	33.73	34.01

Rabbit 7. 2,8-Dioxy-6-methylpurine.

DAY	WEIGHT	URINE			DIET PER DAY: 200 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	
	kilos	*cc.*		*gram*	
1	1.72	154	1.020	0.49	
2	1.70	160	1.020	0.43	
3	1.68	160	1.022	0.55	Injected 0.3013 gm. 2,8-dioxy- 6-methyl-
4	1.66	150	1.020	0.54	purine = 0.101 gm. N.
5	1.68	146	1.024	0.53	
6	1.68	136	1.022	0.53	

Rabbit 8. 2,8-Dioxy-6-methylpurine.

DAY	WEIGHT	URINE				DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Allantoin per period	
	kilos	*cc.*		*gram*	*gram*	
1	2.12	138	1.026	0.63	0.0744	
2	2.14	146	1.020	0.64		
						3d day:
3	2.12	110	1.022	0.70	0.0628	Injected 0.3219 gm. 2,8-dioxy-6-
4	2.20	100	1.028	0.67		methylpurine = 0.108 gm. N.
5	2.14	172	1.020	0.68	0.0596	
6	2.14	146	1.022	0.55		

2,8-Dioxy-9-methylpurine.

After the introduction of this purine, rabbit 10 showed no diuresis, in agreement with previous observations on this compound (Mendel and Kahn, 1913). No toxic symptoms were apparent.

Rabbit 9 was injected with this compound and the allantoin output determined. The allantoin excreted during the experi-

mental period exceeds that of the fore period by 0.022 gram, yet exceeds that of the after period by only 0.008 gram. Whether this increase represents a conversion of the injected purine to allantoin, or is merely a rise to a higher level, due, perhaps, to a stimulating effect of the injection, is difficult to decide. On the basis of the allantoin output in the after period the latter hypothesis seems more probable.

Rabbit 10. 2,8-Dioxy-9-methylpurine.

DAY	WEIGHT	URINE			DIET PER DAY: 200 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	
	kilos	*cc.*		*gram*	
1	1.54	192	1.020	0.58	
2	1.54	174	1.016	0.49	
3	1.52	166	1.030	0.77	3d day: Injected 0.3319 gm. 2,8-dioxy-9-methylpurine = 0.112 gm. N.
4	1.54	170	1.024	0.70	
5	1.52	222	1.016	0.67	
6	1.50	172	1.024	0.62	

Rabbit 9. 2,8-Dioxy-9-methylpurine.

DAY	WEIGHT	URINE				DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Allantoin per period	
	kilos	*cc.*		*gram*	*gram*	
1	1.52	192	1.016	0.40	0.086	
2	1.50	158	1.020	0.47		
3	1.48	136	1.030	0.59	0.1076	3d day: Injected 0.3511 gm. 2,8-dioxy-9-methylpurine = 0.118 gm. N.
4	1.50	160	1.030	0.55		
5	1.50	198	1.016	0.62	0.100	
6	1.44	180	1.020	0.53		

2,8-Dioxy-6,9-dimethylpurine.

Rabbit 11, after receiving this purine, gave no increase in the total nitrogen output, although 0.121 gram of purine nitrogen was injected. No diuretic effect was exhibited in this or in later experiments, confirming previous observations on this compound (Mendel and Kahn, 1913). The experiment was discontinued on the fifth day because of an injury to the animal. It is given here merely to show the peculiarity in the behavior of the nitrogen elimination.

This same effect was exhibited by rabbit 13, after it was injected with this compound. Two injections were made, but in neither case did an increase of nitrogen over the previous day occur. On the contrary, after the injections there was a decrease of the total nitrogen which subsequently increased until it was above the normal. This would seem to indicate a deposition of the purine in some tissue or organ of the body, possibly the kidneys. That there was some injury to the kidney is further indicated by the fact that both in this experiment and in the experiment on rabbit 12, the urine contained blood on the day of the injection.

In the experiment on rabbit 12, the allantoin output was determined. The total nitrogen excreted on the day of the injection fully accounted for the purine nitrogen injected. The allantoin output during the experimental period did not exceed that of the fore period. A slight increase occurred during the after period. This increase amounted to 0.013 gram over the fore period and to only 0.008 gram over the experimental period; hardly enough to warrant a conclusion that the increase was due to a conversion of the purine into allantoin.

Another observation with this compound is described with the discussion of the experiment on rabbit 14, page 100.

Rabbit 11. 2,8-Dioxy-6,9-dimethylpurine.

DAY	WEIGHT	URINE			DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	
	kilos	*cc.*		*gram*	
1	1.74	176	1.020	0.50	
2	1.74	126	1.020	0.55	
					3d day:
3	1.80	184	1.024	0.57	Injected 0.3891 gm. 2,8-dioxy-6,9-dimethyl-
4	1.76	192	1.018	0.55	purine = 0.121 gm. N.

Rabbit 13. 2,8-Dioxy-6,9-dimethylpurine.

DAY	WEIGHT	URINE			DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	
	kilos	*cc.*		*gram*	
1	1.84	124	1.020	0.50	
2	1.82	180	1.016	0.50	
3	1.82	180	1.020	0.57	
					4th day:
4	1.78	180	1.016	0.40	Injected 0.5338 gm. 2,8-dioxy-6,9-di-
5	1.70	224	1.014	0.39	methylpurine = 0.166 gm. N. Urine con-
6	1.68	184	1.010	0.52	tained blood on this day.
7	1.68	152	1.016	0.67	
8	1.68	176	1.024	0.65	
9	1.70	146	1.020	0.69	
10	1.70	166	1.024	0.67	
					11th day:
11	1.70	176	1.018	0.58	Injected 0.4705 gm. of 2,8-dioxy-6,9-di-
12	1.70	150	1.024	0.53	methyl-purine = 0.147 gm. N.

Rabbit 12. 2,8-Dioxy-6,9-dimethylpurine.

DAY	WEIGHT	URINE				DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Allantoin per period	
	kilos	*cc.*		*gram*	*gram*	
1	1.66	120	1.020	0.52	0.103	
2	1.68	162	1.018	0.52		
3	1.68	170	1.030	0.62	0.108	3d day: Injected 0.3097 gm. of 2,8-dioxy-6,9-dimethylpurine = 0.096 gm. N. 3d day: Animal passed blood in urine.
4	1.62	108	1.022	0.52		
5	1.66	162	1.018	0.70	0.116	
6	1.64	160	1.012	0.49		

Control experiment.

Rabbit 18 served as a control of the nitrogen output from a normal animal on the diet employed. The total nitrogen values for the first four days were very constant. On the fifth day, an injection of physiological saline containing the amount of sodium hydroxide used as a solvent in the other experiments, was made. The total nitrogen value remained approximately normal, at least no increase occurred. After the fifth day, the animal refused to eat all of the food and, as a consequence, there was a slight increase of the total nitrogen output. The duration of most of the experiments reported here did not exceed nine days; furthermore, this factor of a lack of food does not enter into them. Hence we may conclude that the diet was adequate for the length of the experiments and that no disturbance of the nitrogen excretion resulted merely from the injection of sodium hydroxide.

Rabbit 18. Control.

DAY	WEIGHT	URINE			DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	
	kilos	*cc.*		*gram*	
1	1.60	120	1.012	0.38	
2	1.56	208	1.012	0.39	
3	1.60	126	1.020	0.38	
4	1.60	154	1.018	0.35	
					6th day:
5	1.58	150	1.012	0.30	Injected 25 cc. of physiological saline con-
6	1.50	200	1.020	0.33	taining 1 cc. of 10% sodium hydroxide.
7	1.42	80	1.026	0.59	Refused to eat all of daily diet after 6th
8	1.38	34	1.040	0.67	day.

2,6,8-Trioxypurine (uric acid).

Because of the paucity of literature on the subject it seemed advisable to demonstrate the conversion into allantoin of a purine related to the synthetic purines studied and injected under comparable conditions into the rabbit. The work of Wiechowski (1908) alone gives figures for both allantoin and uric acid output after subcutaneous injection of uric acid into the rabbit. Bendix and Schittenhelm (1904) and Burian and Schur (1901) give figures for uric acid eliminated unchanged after subcutaneous injection of uric acid into rabbits. Accordingly uric acid was injected into rabbits 20, 21 and 23. In rabbit 20, taking an average of the fore and after periods as the normal output of allantoin, the increase of that excreted in the experimental period amounted to 35 per cent of the uric acid injected.

The experiment on rabbit 21 was a repetition of the above, except that in this case the uric acid was injected as a lithium salt. The excretion of allantoin during the experimental period, in excess of the average of the fore and after periods, amounted to 45 per cent of the uric acid injected.

Rabbit 23 was also injected with uric acid dissolved in lithium carbonate solution and the output of uric acid and purine bases, other than uric acid, was determined. The latter showed no appreciable variation in the fore and experimental period and

will therefore be disregarded. The data of the after period as a whole have not been drawn into comparison, as the animal was abnormal during this time. The experimental period was of three days duration; ample for the absorption of all of the uric acid injected. The total nitrogen output rose on the third and fourth days above that which could be accounted for by the uric acid nitrogen introduced.

The increase of uric acid in this experiment amounted to 12.2 per cent of that injected. This figure for the unchanged uric acid is higher than that obtained by Wiechowski (5.6 per cent), but agrees with those obtained by Bendix and Schittenhelm (18 per cent) and by Burian and Schur (16 per cent) after subcutaneous injections of uric acid into the rabbit. On the other hand, the figures for the allantoin output (35–45 per cent) are lower than those obtained by Wiechowski (53 per cent of the uric acid injected) under similar conditions.

The work of Starkenstein (1907) may throw some light on the cause of the wide variations; for he found that uric acid is not altogether an inert substance. When administered subcutaneously into rabbits, it may lead to metabolic disturbances. Eckert (1913) has recently shown that when uric acid is injected in piperazine solution intravenously into rabbits, concrements may form in the kidneys; however, this was not found to be the case when subcutaneous injections were made.

Rabbit 20. 2,6,8-Trioxypurine (uric acid).

DAY	WEIGHT	URINE				DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Allantoin per period	
	kilos	*cc.*		*gram*	*gram*	
1	2.28	144	1.020	0.72	0.034	
2	2.24	174	1.020	0.59		
3	2.28	116	1.022	0.50	0.184	3d day: Injected 0.4425 gm. of uric acid = 0.152 gm. N, equivalent to 0.420 gm. allantoin.
4	2.24	142	1.018	0.84		
5	2.16	180	1.020	0.88	0.044	
6	2.14	134	1.024	0.68		

Rabbit 21. 2,6,8-Trioxypurine (uric acid).

DAY	WEIGHT	URINE				DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Allantoin per period	
	kilos	*cc.*		*gram*	*gram*	
1	1.84	180	1.016	0.55	0.0825	
2	1.82	138	1.020	0.68		
3	1.82	110	1.022	0.56		
4	1.84	160	1.030	0.63	0.272	Injected 0.3707 gm. uric acid as lithium salt = 0.127 gm. N, equivalent to 0.350 gm. allantoin.
5	1.88	160	1.032	0.45		
6	1.88	136	1.038	0.55		
7	1.90	150	1.026	0.48	0.151 (For 3 day period)	
8	1.88	150	1.022	0.47		

Rabbit 23. 2,6,8-Trioxypurine (uric acid).

DAY	WEIGHT	URINE					DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Uric acid per period	Remainder purine N	
	kilos	*cc.*		*grams*	*gram*	*gram*	
1	2.22	130	1.030	0.82	0.029	0.006	
2	2.22	170	1.030	0.81	(3 day period)	(3 days)	
							3d day:
3	2.22	160	1.026	0.96	0.076	0.0065	Injected 0.3851 gm. uric acid as lithium salt = 0.132 gm. N.
4	2.24	150	1.024	1.06		(3 days)	
5	2.14	80	1.042	0.86			
							6th day:
6	2.18			0.76	0.009	none	Urine scanty and bloody
7	2.22	80	1.030	0.87	(3 day period)		

2,6-Dioxypurine (xanthine).

In view of the results on the allantoin output after injections of the synthetic 2,8-dioxypurine and of the absence in the literature of data dealing with the output of allantoin, after injection of its naturally occurring isomer 2,6-dioxypurine, a study was made of the allantoin excretion when this compound was injected subcutaneously into the rabbit. Lewinthal (1912) injected an impure preparation of xanthine into the ear vein of a rabbit recovering 38 per cent unchanged and 40 per cent as uric acid. Hunter and Givens (1914) introduced a pure preparation into the monkey. As a result of introducing this compound into rabbit 14, the experimental period showed an increase of 0.009 gram over the fore period and an increase of only 0.003 gram over the after period. Both are negligible. From this experiment we must conclude that the introduction of 2,6-dioxypurine did not affect the allantoin output.

The same animal, because of its very uniform total nitrogen output, was given an injection of 2,8-dioxy-6,9-dimethylpurine after the end of the above experiment. In agreement with former observations, no increase of the total nitrogen excretion took place on the day of the injection or on the succeeding days.

The experiment on rabbit 22 is a repetition of that on rabbit 14. Here, however, the xanthine was injected as a lithium salt. Contrary to the former result, there is an indication of an increased allantoin output in this experiment. Taking the figure of the fore period as the normal, the excess of the allantoin excreted during the experimental period amounted to 22.8 per cent of the injected xanthine.

It will be seen on inspection of the protocol of this experiment that the nitrogen rose on the day of the injection, and remained throughout the experiment at a higher level than during the fore period. There are two possible explanations of this phenomenon: it was either due to a slow obsorption of the xanthine, or to a disturbance of metabolism caused by the xanthine injection. If we consider the increase of allantoin in the after period as coming from the xanthine injected, which is quite unlikely, the amount of allantoin excreted in the experimental and after periods, above the output of the fore period as a normal, would be 39 per cent of the injected xanthine.

The behavior of xanthine in the organism of the rabbit needs further investigation, as the contrary results obtained in the experiments given above indicate, and from the fact that there are no published records of experiments of this sort, so far as the author knows.

The output of uric acid and purine bases was determined in rabbit 24 after the injection of xanthine in a lithium carbonate solution. Considering the average of the fore and after periods as the normal output of uric acid in this experiment, the increase during the experimental period amounted to 4.6 per cent of the injected xanthine (computed as uric acid). This points to uric acid as an intermediary product of the conversion of xanthine to allantoin. Figured in the same manner, the experimental period showed an increase of purine bases other than uric acid which, if we consider the increase due to xanthine, was 16.8 per cent of

Rabbit 14. 2,6-Dioxypurine (xanthine).

DAY	WEIGHT	URINE				DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Allantoin per period	
	kilos	*cc.*		*gram*	*gram*	
1	1.82	128	1.016	0.47	0.92	
2	1.84	142	1.020	0.71	(Period 2	
3	1.86	142	1.020	0.58	days)	
4	1.82	150	1.016	0.62	0.101	4th day: Injected 0.4898 gm. xanthine = 0.180 gm. N.
5	1.84	148	1.018	0.59		
6	1.80	226	1.016	0.55	0.098	
7	1.76	190	1.014	0.55		

2,8-Dioxy-6,9-dimethylpurine.

8	1.76			0.51		8th day: Injected 0.7994 gm. 2,8-dioxy-6,9-dimethylpurine = 0.249 gm. N.
9	1.72	210	1.022	0.46		
10	1.74	172	1.016	0.38		
11	1.74	132	1.020	0.37		
12	1.74	180	1.018	0.42		

Rabbit 22. 2,6-Dioxypurine (xanthine).

DAY	WEIGHT	URINE				DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Allantoin per period	
	kilos	*cc.*		*gram*	*gram*	
1	1.98	176	1.014	0 55	0.0555?	
2	1.98	126	1.028	0.52		
3	2.00	140	1.024	0.56		
						4th day:
4	2.20	220	1.020	0.89	0.179	Injected 0.5187 gm. of xanthine
5	2.20	200	1.014	0.67		as lithium salt = 0.191 gm. N.
6	1.96	204	1.020	0.77		
7	1.98	170	1.026	0.69	0.144	
8	1.94	170	1.020	0.63		
9	1.96	180	1.018	0.67		

Rabbit 24. 2,6-Dioxypurine (xanthine).

DAY	WEIGHT	URINE					DIET PER DAY: 225 gms. carrots 20 gms. oats
		Volume	Specific gravity	Total N	Uric acid N per period	Remainder Purine N	
	kilos	*cc.*		*grams*	*gram*	*gram*	
1	2.34	130	1.020	0.64	0.0246	0.0022	
2	2.36	140	1.038	0.63	(3 days)	(3 days)	
							3d day:
3	2.38	150	1.024	1.04	0.108	0.0365	Injected 0.5312 gm. xan-
4	2.36	180	1 016	0.72		(3 days)	thine as lithium salt
5	2.40	160	1.030	0.74			= 0.196 gm. N.
6	2.38	166	1 032	0 69	0.029	0 005	
7	2.36	140	1 026	0.61	(3 days)	(3 days)	

the total amount injected. These figures differ greatly from those of Hunter and Givens (1914) on the monkey; they obtained 30 per cent unchanged and the remainder as allantoin and uric acid after subcutaneous injection of xanthine.

DISCUSSION.

Several interesting points are suggested by the above protocols. First, the failure of the rabbit to oxidize a purine so closely related to xanthine and uric acid as is 2,8-dioxypurine is another evidence of the specificity of the enzymes acting on purines.

Second, that the character of the enzyme content of the organs and tissues is a factor closely related to the oxidation of injected purines, is further shown in a comparison of the results obtained after the injection of uric acid and of xanthine. The former never failed to produce an increase of allantoin output from the rabbit. The latter, on the other hand, failed in one experiment and gave positive results in the other. Whether the failure in the first case was due to the form in which the purine was injected or was due to a deposition, cannot at this time be stated. From a review of the literature on purine enzymes, another possibility suggests itself, namely, a wider distribution of the uricolytic enzyme than of the xanthine oxidase in this species. The uricase has been found in both the kidney and the liver of the rabbit, while xanthine oxidase has been found, so far as is known, in the liver only. Hence the possibility of a complete or partial escape from oxidation is not unlikely. Hunter and Givens (1914) observed in the monkey, after parenteral introduction, that "there escapes metabolism more xanthine than any other injected purine except uric acid."

Third, the failure of the methylated purines investigated to increase the allantoin output in rabbits is in line with our knowledge of the difficulty of complete demethylation in the body.

Lastly, the absence of a diuretic effect with the synthetic methylated purines studied would tend to indicate that this physiological action is closely dependent upon the position of the methyl groups in the purine nucleus.

SUMMARY.

2,8-Dioxypurine—an isomer of xanthine—is not converted into allantoin when injected subcutaneously into the rabbit, but is excreted almost quantitatively unchanged in the urine.

Uric acid, when injected subcutaneously into the rabbit, is

excreted in part (35–45 per cent) as allantoin and in part unchanged (12 per cent).

Xanthine, injected subcutaneously into the rabbit, may be excreted partly as allantoin (22–39 per cent), partly as uric acid (5 per cent) and partly unchanged (17 per cent).

The methylated purines, 2,8-dioxy-6-methyl, 2,8-dioxy-9-methyl, and 2,8-dioxy-6,9-dimethylpurine, do not lead to increase of allantoin when injected subcutaneously into the rabbit. There is evidence that 2,8-dioxy-6,9-dimethylpurine injures the kidneys, leading to a retention of nitrogen.

2,8-Dioxy-6-methylpurine may be, in part, isolated unchanged from the urine after subcutaneous injection into the rabbit.

None of the synthetic purines mentioned above cause diuresis in the rabbit.

The writer wishes to acknowledge his indebtedness to Prof. Lafayette B. Mendel for suggesting this problem for study and for helpful suggestions throughout the work, and to Prof. C. O. Johns for aid in the synthesis of the compounds used.

BIBLIOGRAPHY.

Ach: *Arch. f. exp. Path. u. Pharm.*, xliv, p. 319, 1900.
Albanese: *Ibid.*, xliii, p. 305, 1900.
Bendix and Schittenhelm: *Zeitschr. f. physiol. Chem.*, xlii, p. 46, 1904.
Burian and Schur: *Pflügers Archiv*, lxxxvii, p. 326, 1901.
Eckert: *Arch. f. exp. Path. u. Pharm.*, lxxiv, p. 244, 1913.
Hunter and Givens: This *Journal*, xvii, p. 55, 1914.
Johns: *Amer. Chem. Journ.*, xli, p. 63, 1909. *ibid.*, xlv, p. 79, 1910. This *Journal*, ix, p. 161, 1911. *Ibid.*, xi, p. 393, 1912.
Lewinthal: *Zeitschr. f. physiol. Chem.*, lxxvii, p. 259, 1912.
Mendel and Lyman: This *Journal*, viii, p. 115, 1910.
Mendel and Kahn: *Journ. Pharm. and Exp. Ther.*, iv, p. 343, 1913.
Schittenhelm: *Ther. Monatsh.*, xxlv, p. 113, 1910.
Starkenstein: *Arch. f. exp. Path. u. Pharm.*, lxii, p. 27, 1907.
von Schröder: *Ibid.*, xxii, p. 39, 1887.
Wiechowski: *Hofmeister's Beiträge*, xi, 109, 1908.

A STUDY IN DRYING URINE FOR CHEMICAL ANALYSIS.

By WINFRED W. BRAMAN.

(From the Institute of Animal Nutrition of the Pennsylvania State College, State College, Pa., in coöperation with the Bureau of Animal Industry of the United States Department of Agriculture.)

(Received for publication, July 27, 1914.)

In the determination of the heat of combustion of urine it is necessary first to get rid of the large amount of water present. During the drying to remove this water, decomposition takes place giving rise to a loss of substance and therefore to a loss of energy.

Many suggestions[1] have been made as to the method of drying. Farkas and Korbuly[2] made an extensive study of the calorimetry of urine. They reviewed the work done by Rubner, Kellner and others and concluded that the smallest loss of energy occurred when drying the urine in vacuum at room temperature, without any addition whatever. They further concluded that it mattered very little in practical calorimetry whether Rubner's correction of 5.45 calories, or Krummacher's correction of 6.518 calories per gram of nitrogen lost, was used. Most of this work was done with the urine of omnivora and carnivora and but little with that of herbivora. In every case particular attention has been paid to the loss of nitrogen with almost no attention to the loss of carbon.

The plan of this experiment was to dry steer's urine in such a manner that the loss of carbon as well as the loss of nitrogen could be determined and therefore possibly some relationship established between the two. Further, urines were to be dried by different methods as regards temperature, etc., and energy determinations made on the residues so as to see if a change in the loss of carbon and nitrogen made a definite change in the energy value.

[1] Glikin: *Kalorimetrische Methodik*, p. 67; Neuberg: *Der Harn*, ii, p. 1323.
[2] Farkas and Korbuly: *Arch. f. d. ges. Physiol.*, civ, p. 564.

In the metabolism experiments it was impracticable to make analyses daily. Each urine used for analysis was a composite sample covering the urine of ten successive days, the animal being fed a definite ration. These samples were preserved in rubber-stoppered bottles, with the addition of a few cubic centimeters of chloroform. The bottles were kept in a refrigerator. Always to make sure that the compositing was accurate, total nitrogen determinations were made upon the daily urines on the days of compositing. The true average per cent of total nitrogen for the ten days always agreed very closely with the per cent of total nitrogen determined upon the composite.

Two methods of drying were used. The one was to dry 10 gram charges, in platinum capsules in Hempel desiccators at about 5 mm. vacuum, using concentrated sulphuric acid as the drying agent. Samples could thus be dried at any desired room temperature.

The second method was to dry charges of 5–10 grams of urine in platinum boats or capsules in a current of dry NH_3- and CO_2-free air. This current after passing over the urine bubbled through a known amount of $\frac{5N}{14}$ H_2SO_4. Then it was dried by concentrated H_2SO_4, and drawn through a train of absorption tubes to remove CO_2. The NH_3 was determined in the standard acid and the CO_2 by absorption with soda lime.

The glass tubes carrying the platinum dishes with charges were in a special form of water oven, the ends of the glass tubes projecting to either side of the oven, the temperature of which was electrically regulated. Also the oven could be turned over and the chamber about the glass tubes packed with ice so as to produce low temperatures.

This method of drying allowed the determination of the total loss of NH_3 and of CO_2 on drying. It gave no information as to whether the NH_3 and CO_2 were present as such in the urine or were derived from some decomposition during drying. To test this in the fresh urine, NH_3 was determined by Steele's modification of Folin's method and the free NH_3 by Folin's method modified by the addition of some NaCl. In connection with the determination of free NH_3, an effort was made to determine the free CO_2, by conducting the air current, dry and NH_3-free,

through an absorption train, thus absorbing the CO_2 in the soda lime.

In the routine analytical work connected with the experiments with the respiration calorimeter, the total carbon and organic hydrogen were determined. This was done by burning in a quartz combustion tube, with copper oxide, the residues dried in an air current at 55°–60°C. The carbon thus obtained was corrected by adding the carbon of the CO_2 lost on drying and the hydrogen was corrected by adding the hydrogen of the NH_3 lost on drying. This method did not tell whether or not all of the carbon lost on drying consisted of CO_2.

It was decided to try burning fresh urine in the usual combustion furnace tube and thus get the total carbon and total hydrogen, including that of the water, at once. These combustions were made in a quartz tube containing a 50 cm. layer of copper oxide and beyond at the exit end a 20 cm. layer of reduced copper in spiral or wire-gauze form. The charge of 4–5 grams fresh urine was weighed by difference into a porcelain boat 12 cm. long containing, at first some ignited asbestos, but later a strip of reduced copper gauze. The charge was put into the boat as it lay in the combustion tube, projecting a little for the time being. All charges of urine were weighed by difference from a small 30–40 cc. flask, rubber stoppered, the stopper carrying a medicine-dropper pipette.

The absorption train at the exit end of the combustion tube consisted for the water, of a Peligot tube containing concentrated H_2SO_4 followed by an acid pumice U-tube. Later a Winkler-Kyll absorption bulb was used. Some such form of apparatus was necessary to avoid sucking back of acid into the combustion tube when any considerable amount of water was absorbed. For the absorption or CO_2, one or two soda lime U-tubes with two acid U-tubes were used.

After putting in the charge and a copper spiral after it, the quartz tube was heated for two-thirds of its length, beginning at the exit end, until red hot. Then a current of dry CO_2-free air was forced over the urine through the heated tube into the absorption train. The tube immediately about the charge had to be kept cool to the finger tips. In about one and one-half hours the urine charge was nearly dry and most of the water had gone over into the acid absorption bulb, or had condensed about the

exit end of the combustion tube. At this time the remaining third of the combustion tube was heated gradually to redness. After about all of the condensed water had left the exit end of the tube, the heat under the reduced copper spiral was turned off and the stream of air was changed to a stream of oxygen. This

TABLE I.

Total carbon and total hydrogen in steer's urine. Percentage computed on fresh substance.

SAMPLE NO.	TOTAL CARBON, CARBON IN RESIDUE + CARBON LOST ON DRYING	TOTAL CARBON BY BURNING FRESH URINE	TOTAL HYDROGEN BY BURNING FRESH URINE
	per cent	*per cent*	*per cent*
1162	1.953	1.961	10.548
	1.989	1.904	10.525
		2.099	
		2.066	
1189	1.278	1.338	10.859
	1.278	1.292	10.866
		1.336	10.712
1215		1.406	10.772
		1.370	10.775
		1.434	10.793
1239		1.505	10.761
		1.530	10.869
		1.484	10.762
1274	2.020	2.080	10.572
	2.060	2.047	10.527
1300		1.668	10.694
		1.653	10.571
		1.619	10.642
			10.630
			10.663
1325		1.555	10.701
		1.662	10.708
		1.698	10.684
		1.609	10.684
1369		1.784	10.671
		1.773	10.640

was continued for some time after the exit end of the tube was dry and until oxygen was detected at the end tube of the absorption train. The reduced copper successfully decomposed the oxides of nitrogen and held the chlorine of the volatile salts. The method gave accordant results as shown in table I.

The percentage of total carbon by the new method compared with the total carbon computed from the loss as CO_2 and the carbon in the residue shows that the total loss of carbon on drying was from CO_2.

TABLE 2.

Analyses of steer's urine. Percentage computed on fresh substance.

SAMPLE NUMBER	1162		1189		1215		1239		1274	
Daily feed	3 kg. starch 7.5 kg. alfalfa hay		1.2 kg. starch 3 kg. alfalfa hay		1.7 kg. starch 4.25 kg. alfalfa hay		0.8 kg. starch 2 kg. alfalfa hay		9 kg. alfalfa hay	
By direct determination.										
	N	C	N	C	N	C	N	C	N	C
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Total	0.934	1.995	0.711	1.304	0.691	1.406	0.899	1.506	1.322	2.052
Loss as NH_4 compds. including NH_3	0.415		0.401		0.334		0.146		0.074	
Loss as free NH_3	0.138		0.156		0.125		0.073		0.034	
Loss on drying in air current at 55°-60°C.	0.441	0.524	0.432	0.333					0.097	0.270
Loss on drying in air current at 10°-20°C.			0.427	0.363	0.343	0.254				
Loss on drying in air current below 10°C.			0.424	0.412						
Indirectly by computation from determinations on residues.										
Loss on drying in vacuum at 20°C.	0.412	0.474	0.427	0.312	0.371	0.362	0.150	0.204	0.058	0.179
Loss on drying in vacuum, while frozen					0.326	0.302			0.061	0.184

Briefly the determinations made were the following: in fresh urine, total nitrogen, total carbon, total hydrogen, nitrogen as NH_3 and NH_4 together, and nitrogen as NH_3; (CO_2 as such was tried but not successfully); in the residues dried in vacuum in Hempel desiccators, carbon, nitrogen and energy; in the drying in air current, the loss of carbon as CO_2 and the loss of nitrogen as

NH_3; in the residues dried in air current, carbon, nitrogen and energy.

The nitrogen was determined always by the Kjeldahl method. The energy was determined in an Atwater-Hempel bomb, using the Fries adiabatic[3] device.

The drying in Hempel desiccators was tried at room temperature of about 20°C., and also at a little above 0° after having first frozen the urine charges. The drying in air current was tried at 55°–60°C, at 10°–20°C. and also at below 10°.

The summarized results are shown in Table 2.

The losses on the samples dried in Hempel desiccators are computed by difference. The percentages are the averages of from two to six determinations.

The table shows that in the case of the nitrogen the losses by the different methods differed only slightly. Further the determinations of the nitrogen of free NH_3 and of NH_4 compounds by Steele's modification of Folin's method gave in every case an amount of nitrogen only slightly less than the greatest loss on drying, even at 55°–60°. Thus we have,

URINE NO.	GREATEST LOSS ON DRYING, PER CENT N	FREE NH_3 AND NH_4 COMPOUNDS PER CENT N
1162	0.441	0.415
1189	0.432	0.401
1215	0.371	0.334
1239	0.150	0.146
1274	0.097	0.074

Since this method liberates no NH_3 from urea, uric acid or hippuric acid, it is fair to conclude that the greater part of the nitrogen lost came from free NH_3 and NH_4 compounds. In every case some free NH_3 was found.

With the Folin method for free NH_3 some difficulty was found in that not only free NH_3 but also NH_3 from NH_4 compounds was taken out of the urines during the determinations. It was found that the addition of 10 grams of sodium chloride to a charge of 5 grams of urine prevented some ammonium salts from breaking up.[4]

[3] Fries: *Journ. Amer. Chem. Soc.*, xxxiv, p. 643.

[4] From unpublished results by D. C. Cochrane of this Institute.

This subject is now being taken up in this laboratory.

In respect to the carbon, the losses on drying were somewhat greater for the higher temperatures. The variation was greater than with the loss of nitrogen. With each urine the determination of free CO_2 was tried. Always some CO_2 was found but no results were obtained which checked.

The fact that all of the carbon that was lost was CO_2 and that nearly all of the nitrogen lost was from NH_4 compounds and free NH_3, points decidedly to the presence of ammonium carbonates. Also, from the fact that always more carbon was lost than would be required to combine with the nitrogen lost for the formation of normal carbonate, it seems reasonable to suppose some bicarbonate was present in the urine.

At the time it was found impossible to dry enough samples so that sufficient energy determinations could be made to attempt any definite conclusions regarding the relation of differences in losses to differences in energy. Energy determinations were made upon some of the dried residues. Energy values of residues obtained by drying under differing conditions were determined only on urine 1274, the sample showing the smallest loss of nitrogen and carbon on drying.

Sample No. 1274	Calories per gram fresh substance
In residues obtained by	
drying in air current 55°–60°	186.89
drying in vacuum at 20°	194.72
drying in vacuum after freezing	193.49

The above figure 186.89 is the mean of the two results differing by six calories which is not a good agreement. The values for the residues dried at 20° and while frozen are the averages of three and two determinations respectively, differing by less than one calorie.

Determinations for urea and hippuric acid were not made, owing to lack of time and equipment. Undoubtedly these would have helped in determining any relation between carbon and nitrogen, knowing the free CO_2 and NH_3.

According to J. Tereg,[5] the nitrogen content of cattle urine is principally in urea and hippuric acid, in all cases the urea being

[5] Tereg: *Ellenberger's Physiologie der Haussäugthiere*, i, p. 390.

the main carrier of nitrogen. Even with urines rich in hippuric acid fully two-thirds of the total nitrogen belongs with the urea. There seemed to be no relation between the urea content and the hippuric acid content.

Quite different from the above are the results found with urines 1162, 1189 and 1215. In these urines only 55.5, 43.6 and 51.6 per cent respectively, of the total nitrogen was present in some form other than NH_4 compounds and free NH_3. In urines 1239 and 1274, 83.7 and 94.4 per cent respectively, of the total nitrogen was present in some form other than NH_4 compounds and free NH_3. These results show plainly that in some urines one-half of the total nitrogen can be in the form of NH_4 compounds and free NH_3.

As regard the relation of the different feeds to the quantities of carbon and nitrogen obtained, not much can be stated. The small loss of nitrogen in urines 1239 and 1274, a very light ration of starch and hay and a heavy ration of hay alone, respectively, indicates the presence in the urine of organic compounds not so readily broken up by a temperature as high as 60°C. or by bacterial or enzyme action. It is to be noted that the total nitrogen in urine 1274 is much higher than of any of the other urines.

The method of making up the samples and the time of making determinations should be considered. The first aliquot of each composite sample was ten days old, the second, nine days old and so on, before any determinations on the composite sample could be started. The true average per cent of daily nitrogen showed there was no loss of nitrogen from the composite but did not detect any rearrangement of nitrogen compounds within the urine during the compositing. Very recently Dehn and Hartman[6] have studied preservatives for urine and show how difficult it is to preserve urine for chemical analysis. In regard to the urines used here, it is a question as to what would have been the result had the fresh daily urines been used for analysis instead of the composite samples. Such an undertaking would be impossible without a special force and equipment.

[6] Dehn and Hartman, *Journ. Amer. Chem. Soc.*, xxxvi, p. 409.

SUMMARY OF CONCLUSIONS.

The data presented below show

1. That in some urines from herbivora, on drying, the loss of nitrogen from NH_4 compounds and free NH_3 may be as high as 50 per cent of the total nitrogen of the urine.

2. That the carbon lost consists of CO_2 and is more than enough in quantity to combine with the NH_3 lost to form normal carbonates.

3. That the loss of nitrogen comes mainly from the decomposition of ammonium carbonate and not from the decomposition of urea or hippuric acid.

4. That the loss of carbon comes partly from the normal carbonate and partly from the bicarbonate.

5. That the total carbon and total hydrogen, including that of water, can be determined directly by combustion in a quartz combustion tube, using the ordinary combustion furnace.

THE EXCRETION OF CREATININE BY NORMAL WOMEN.

By MARTHA TRACY and ELIZABETH E. CLARK.

(From the Department of Chemistry, Woman's Medical College of Pennsylvania, Philadelphia, Pa.)

(Received for publication, July 30, 1914.)

The active investigation of the problems involved in creatinine and creatine metabolism makes all data in regard to these substances of interest and value.

The published reports in regard to the excretion of creatinine by women on creatinine- and creatine-free diets include seven cases of insane women reported by Folin;[1] twenty-six cases, of whom twenty-five were insane and one a convalescent typhoid reported by Benedict and Myers;[2] and seven cases, of whom three were normal women and four infirmary patients, reported by Krause.[3]

Of the above forty cases, therefore, only three were strictly normal individuals. While the evidence offered by Folin and others indicates that the nitrogen metabolism is not necessarily significantly altered in mental diseases it would appear to be more satisfactory if normal standards for women as well as for men might be established by observations upon strictly normal individuals.

With a view, therefore, to adding data in this interesting field the present report is offered as a matter of record.

The subjects of the investigation, women medical students, were not subjected to rigid physical examination, but were all pursuing their active college work, and might properly be considered normal individuals. All were upon a strict creatinine- and creatine-free diet for two days or longer.

[1] Folin: *Amer. Journ. of Insanity*, lx, p. 699, 1904, and lxi, p. 299.
[2] Benedict and Myers: *Amer. Journ. of Physiol.*, xviii, 1907.
[3] Krause: *Quart. Journ. of Exp. Physiol.*, iv, 1911.

Creatinine excretion in normal women on a creatinine-free diet.

NUMBER	NAME	WEIGHT	HEIGHT	CREATININE	CREATININE	CREATINE-N	CREATINE-N GMS. PER KILO CREATININE COEFFICIENT
		kilo	*cm.*	*grams*	*gms. per kilo*	*grams*	
1	A. O.	72.0	172.0	1.71	0.023	0.649	0.0090
2	M. M.	50.0	167.5	1.29	0.025	0.490	0.0098
3	F. W.	61.1	155.0	1.13	0.018	0.429	0.0070
4	C. A. O.	62.2	161.0	1.11	0.017	0.421	0.0067
5	S. F.	80.2		1.08	0.013	0.410	0.0051
6	S. S. M.	60.0	160.0	1.05	0.017	0.339	0.0066
7	E. Y.	55.0	160.0	1.05	0.019	0.399	0.0072
8	M. L.	52.0	154.0	1.04	0.020	0.395	0.0076
9	C. H.	62.0	167.5	1.03	0.016	0.391	0.0063
10	D. V. A.	56.4	155.5	0.95	0.016	0.361	0.0064
11	M. A. B.	56.6	164.0	0.92	0.016	0.349	0.0061
12	M. L. B.	60.4	175.5	0.89	0.014	0.338	0.0056
13	H. C.	48.0	158.0	0.86	0.017	0.326	0.0067
14	S. S.	61.7	165.0	0.84	0.013	0.319	0.0051
15	S. H.	58.0		0.83	0.014	0.315	0.0052
16	R. Y.	70.0	161.0	0.82	0.011	0.311	0.0044
17	C. W.	47.0	154.0	0.80	0.017	0.304	0.0064
18	M. V.	62.0	169.0	0.77	0.012	0.292	0 0047
19	J. L. B.	54.0		0.77	0.014	0.292	0.0054
20	R. W.	51.0		0.76	0.015	0.288	0.0056
21	T. K.	56.0	152.0	0.69	0.012	0.262	0.0046
22	A. L. G.	59.0		0.66	0.011	0.250	0.0042
23	T. R. H.	70.0	170.0	0.66	0.0094	0.250	0.0035
24	F. H.	58.0	160.0	0.64	0.011	0.243	0.0044
25	A. O. X.	51.2	155.5	0.58	0.011	0.220	0.0043
26	E. R.	51.5	159.0	0.53	0.010	0.201	0.0039
Average				0.90	0.015	0.342	0.0058
Folin's average				0.812	0.0152	0.308	0.0057
Benedict and Myers				0.739	0.0134	0.280	0.0050
Krause's normal				1.186		0.441	0.0057
Krause's infirmary				0.480		0.178	0.0034
Krause's normal pregnant						0 253	

Our cases show a slightly higher absolute creatinine excretion than the cases of Folin, of Benedict and Myers, and the infirmary cases of Krause, though slightly lower than Krause's normal cases. Our average creatinine coefficient, however, is practically identical with those of the other investigators, with the exception of Krause's infirmary cases and one normal pregnant woman, which are considerably lower.

Shaffer[4] finds a creatinine coefficient (mgm. creatinine nitrogen per kilo of body weight) of 8–11 normal for men. He considers the urinary creatinine an index of muscle catabolism and believes that sex *per se* has no influence on the excretion of this substance, but that women will show a creatinine coefficient comparable to that of men provided the muscular efficiency and amount of adipose tissue is the same.

Our figures show a creatinine coefficient below 8, with the exception of cases 1 and 2. In both of these individuals there is unusual muscle development and control through gymnastic work.

We believe the muscular efficiency of psychopathic patients likely to be below par, as these women as a rule have no definite muscular work to perform. The average student in a professional school, also, shows a tendency to confine herself to sedentary occupation and to neglect physical exercise, and her muscular efficiency may therefore become reduced. Individual women, however, who through training have maintained a good muscle tone, appear to excrete creatinine in amounts comparable with men. Additional figures are desirable in relation to this point.

In view of the suggestive work of Krause in regard to the excretion of creatine by normal women at certain periods of the menstrual cycle, we propose to continue our investigation along these lines for a future report.

[4] Shaffer: *Amer. Journ. of Physiol.*, xxiii, 1908.

STUDIES IN CARBOHYDRATE METABOLISM.

VII. THE INFLUENCE OF SUBCUTANEOUS INJECTIONS OF DEXTROSE AND OF CALCIUM LACTATE UPON THE BLOOD SUGAR CONTENT AND UPON TETANY AFTER THYREOPARATHYROIDECTOMY.

By FRANK P. UNDERHILL and NORMAN R. BLATHERWICK.

(*From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, Conn.*)

(Received for publication, August 3, 1914.)

In a recent communication[1] it has been pointed out that during the tetany resulting from extirpation of the thyroids and parathyroids in dogs a condition of marked hypoglycaemia, without glycosuria, obtains. This fact indicates an intimate relationship of the thyreoparathyroid mechanism with processes concerned in the intermediary metabolism of carbohydrates. Before a direct relationship can be considered firmly established, however, it is essential to demonstrate whether the hypoglycaemia may be merely an *effect* produced by the violent muscular activity or the increased temperature called forth as a result of the disarrangement of some other mechanism induced by thyreoparathyroidectomy.

It is also conceivable that the hypoglycaemia may be the *cause* of the tetany. Such an hypothesis, however, is highly improbable; for in other conditions of marked hypoglycaemia, as in hydrazine and phosphorus poisoning, tetany is not a characteristic symptom. On the other hand, if one reasons that hypoglycaemia is merely an effect of tetany there is little in the literature to support such an assumption, for in the exceedingly severe muscular activity initiated by strychnine glycosuria and hyperglycaemia result. There is also no evidence that increased temperature causes hypoglycaemia, indeed hyperglycaemia may result. Upon theoretical grounds it is therefore probable that

[1] Underhill and Blatherwick: This *Journal*, xviii, p. 87, 1914.

hypoglycaemia in the case under discussion is neither the cause nor the effect of the tetany provoked by thyreoparathyroidectomy. In other words, one is forced to the conclusion that the effect of this operation is manifested in at least two distinct directions. In the first place an effect upon the nervous system is evident as indicated by the production of tetany, and, secondly, carbohydrate metabolism is influenced, hypoglycaemia being the manifestation. From an inspection of the data in the tables below it may be seen that although the blood sugar is always low during an attack of tetany the diminution in blood sugar content begins before there is any suggestion of tetany. Experimentally and theoretically there is agreement for the view that the hypoglycaemia is not an effect of the tetany.

Methods. The animals employed for these experiments were full-grown well-fed dogs that were maintained in a condition of inanition in order to eliminate any conflicting factors likely to be introduced with the food. The thyroids and parathyroids were all apparently removed, with aseptic precautions, under ether anaesthesia only. Blood sugar was estimated by the method of Forschbach and Severin,[2] blood being drawn by puncture of the ear veins, about 20 drops being employed for duplicate determinations. Sugar and calcium solutions were administered subcutaneously and temperature was taken in the rectum.

THE INFLUENCE OF SUBCUTANEOUS INJECTIONS OF DEXTROSE UPON THE BLOOD SUGAR CONTENT AND UPON TETANY AFTER THYREOPARATHYROIDECTOMY.

It is reasonable to assume that if hypoglycaemia is the cause of tetany the resumption of approximately normal blood sugar conditions by dextrose injections should cause the disappearance of tetany and prolong the life of the animal. Some experiments bearing upon this point are given in Tables 1 and 2. In Table 1 the injection of dextrose caused a rise in blood sugar content with an apparent improvement in the symptoms of tetany although the life of the dog was not prolonged. It is possible, however, that the improvement is not to be ascribed to the dextrose in-

[2] Forschbach and Severin: *Arch. f. exp. Path. u. Pharm.*, lxviii, p. 311, 1912.

TABLE 1.

The influence of dextrose injections upon blood sugar content after thyreoparathyroidectomy in the dog.

DATE	SUGAR IN BLOOD		REMARKS
	per cent		
May 25	11.00 a.m.	0.18	Thyroids and parathyroids removed at 11.00–11.30 a.m.
	4.00 p.m.	0.12	Dog normal.
26	9.00 a.m.	0.11	No symptoms.
	4.00 p.m.	0.13	
27	9.00 a.m.	0.11	9.00 a.m. dog had slight tremors of back. 11.30 a.m. the animal showed marked symptoms of tetany. Respiration very rapid. Temperature, 41.8°C.
	11.45 a.m.	0.04	12.45 a.m. *Subcutaneous injection of 30 gm. dextrose in 30 per cent solution (5 gm. per kilo).* Condition about the same. Dog can not stand. Temperature, 40.6°C.
	3.00 p.m.	0.07	Dog walks around room although not normal—all movements are jerky. Respiration, normal. Temperature, 38.6°C.
	7.15 p.m.	0.06	Condition unchanged.
28			9.00 a.m. Dog found dead.

jection at all but to the fact of the passing of the tetany seizures which are well-known to occur with an indefinite periodicity with intervals during which the animal is almost normal.

In Table 2 we have an example in which there is at first marked hypoglycaemia with subsequent restoration to normal conditions without evidence of tetany symptoms (see May 29 and 30). This may indicate an approach to tetany with spontaneous recovery. This period was followed by one with notable hypoglycaemia and tetany. Dextrose injections reëstablished normal blood sugar content without altering the symptoms associated with tetany and they failed to prolong markedly the life of the animal. These experiments therefore indicate that hypoglycaemia can nct be regarded as the cause of the tetany after thyreoparathyroidectomy.

TABLE 2.

The influence of dextrose injections upon blood sugar content after thyreoparathyroidectomy in the dog.

DATE	SUGAR IN BLOOD		REMARKS
	per cent		
May 23	3.00 p.m.	0.18	Thyroids and parathyroids removed at 3.30–4.00 p.m.
29	8.30 a.m.	0.03	Dog appears normal.
	3.30 p.m.	0.07	
30	9.00 a.m.	0.11	Dog appears normal. Temperature, 38.4°C.
	2.00 p.m.	0.09	Condition unchanged.
June 1	9.00 a.m.	0.06	Respiration rather rapid. No other symptoms.
	2.00 p.m.	0.02	Mild tetany. Temperature, 40°C. *Subcutaneous injection of 165 cc. 30 per cent dextrose solution (5 gm. per kilo).*
	5.15 p.m.	0.11	Condition unchanged. Temperature, 40.7°C. Injection of 50 gm. dextrose in 30 per cent solution.
	9.00 p.m.	0.12	Animal walks with difficulty. Jaws set. Labored breathing. Temperature, 36.8°C.
2	9.00 a.m.		Dog found dying with hemorrhage of nose.

THE INFLUENCE OF SUBCUTANEOUS INJECTIONS OF CALCIUM LACTATE UPON BLOOD SUGAR CONTENT AND UPON TETANY AFTER THYREOPARATHYROIDECTOMY.

It has been demonstrated by MacCallum and Voegtlin[3] that tetany induced as a result of thyreoparathyroidectomy may be prevented or inhibited and the life of the animal greatly prolonged by the administration of calcium salts. At the time of the above mentioned experiments the induction of hypoglycaemia as a result of thyreoparathyroidectomy was unknown. It is conceivable that the diminution or disappearance of sugar from the blood stream may act as a contributing factor in the ultimate failure of thyreoparathyroidectomized dogs to survive indefinitely. With this idea in mind experiments (tables 3 and 4)

[3] MacCallum and Voegtlin: *Journ. of exp. Med.*, xi, p. 118, 1909.

TABLE 3.

The influence of calcium lactate upon blood sugar content after thyreoparathyroidectomy in the dog.

DATE	SUGAR IN BLOOD		REMARKS
		per cent	
June 2	2.15 p.m.	0.12	Complete thyreoparathyroidectomy at 2.30–3.00 p.m.
3	9.00 a.m.	0.06	Dog appears normal.
	3.00 p.m.	0.06	
4	9.00 a.m.	0.01	Dog found with marked tremors of legs. Temperature, 38°C. *At 10.00 a.m. injected 10 cc. 5 per cent solution calcium lactate.*
	12.00 m.	0.11	Condition unchanged. *Injected 10 cc. calcium lactate.* Improvement follows very rapidly. Tremors disappear and dog runs around room apparently normal. Temperature, 36.9°C.
	2.30 p.m.	0.07	Dog lively and nearly normal. Temperature, 38.2°C.
	5.30 p.m.	0.05	Bad tremors, especially of legs. Temperature, 38.7°C. *Injected 20 cc. calcium lactate.*
	10.30 p.m.		Dog seems normal—no tremors. Temperature, 38.7°C. *Injected 20 cc. calcium lactate.*
5	9.00 a.m.	0.11	Dog is very weak and tremors are marked. *Injected 10 cc. calcium lactate.*
	12.00 m.	0.11	Condition unchanged. Temperature, 37.6°C.
	5.15 p.m.	0.06	Condition unchanged. Temperature, 36.9°C.
6	10.20 a.m.	0.09	Condition unchanged. Temperature, 37.2°C.
7			Condition unchanged. Temperature, 36.7°C.
8	9.00 a.m.	0.07	Condition unchanged. Temperature, 35.8°C.
9	9.00 a.m.	0.05	Tremors are worse.
10			Conditions are little changed.
11	9.30 a.m.	0.06	Tremors constant but animal not in actual tetany. Jaws set. Extreme weakness. Temperature, 36.2°C. Dog killed.

TABLE 4.

The influence of calcium lactate upon blood sugar content after thyreoparathyroidectomy in the dog.

DATE	SUGAR IN BLOOD		REMARKS
	per cent		
June 6	12.00 m.	0.10	Complete thyreoparathyroidectomy at 12.00–12.00 p.m.
7			Animal seems normal.
8	9.00 a.m.	0.03	Dog found in severe tetany, respiration exceedingly rapid, frothing at mouth. Temperature, 42.6°C. *Injected 20 cc. calcium lactate.*
	12.15 p.m.	0.08	Animal runs around room and drinks water, few tremors, respiration normal. Temperature, 37.2°C.
	4.30 p.m.	0.08	Dog normal in appearance. Temperature, 37.5°C.
9	9.30 a.m.	0.09	Animal appears bright but walks unsteadily. Tremors are noticeable especially of head—has difficulty in drinking owing to chattering of teeth. Temperature, 38.3°C.
	4.30 p.m.	0.09	Condition unchanged.
10			Condition unchanged.
11	9.30 a.m.	0.10	Tremors as before. Jaws set. Temperature, 36.7°C.
12	9.00 a.m.		Dog found dead.

have been carried through to test the influence of subcutaneous injections of calcium lactate upon the blood sugar content and upon the progress of tetany.

Table 3 shows a noticeable tendency toward hypoglycaemia previous to any tetany manifestations (June 3). When hypoglycaemia was most marked (June 4), however, evidences of tetany were present. Three hours after the injection of the calcium salt normal blood sugar content had been restored without checking the symptoms of tetany. A second administration of calcium lactate led to the restoration of normal conditions in the animal. This state was not maintained for an appreciable period; for soon there was evidence of a second falling off in blood sugar content which was followed somewhat later by renewal of

TABLE 5.

The relation of hypoglycaemia to the onset of tetany.

DATE	SUGAR IN BLOOD		REMARKS
		per cent	
June 10	9.30 a.m.	0.09	Complete thyreoparathyroidectomy 9.30–10.00 a.m. Weight 6.6 kilos. Severe hemorrhage during operation.
11	10.00 a.m.	0.06	Dog seems normal.
	3.45 p.m.	0.02	Suggestion of tremors or fright (?). Rapid heart beat. Temperature, 39.5°C.
	5.30 p.m.		Slight tremors in hind legs.
	8.00 p.m.	0.02	Violent tetany. Temperature, 41°C. *Injected 20 cc. calcium lactate.* No immediate effect noted.
12	9.00 a.m.		Dog found dead.

the tetany symptoms. Repeated injections of calcium lactate (June 4 and 5) resulted in bringing about the normal blood sugar content without causing the total disappearance of incipient tetany. Cessation of calcium treatment led to a state of continued hypoglycaemia and a condition of mild tetany, which gradually reduced the animal to a condition where it became desirable to terminate the experiment.

It is apparent from these results that in tetany calcium plays an important rôle in maintaining the equilibrium of the sugar regulating mechanism for when there is a sufficiency of calcium blood sugar content is normal. Conversely, blood sugar content tends to become low when there is an apparent deficiency of calcium.

The data of Table 4 show that after the initiation of hypoglycaemia and the inception of tetany a single injection of calcium lactate may restore and maintain blood sugar content almost at normal and ward off actual tetany for a period of two days although life may not be prolonged.

Table 5 is included since its data demonstrate the existence of hypoglycaemia previous to the initiation of actual tetany.

SUMMARY.

From the experiments detailed above it is indicated that

1. Hypoglycaemia resulting from thyreoparathyroidectomy is neither the cause nor the effect of the accompanying tetany; for although dextrose injections restore blood sugar content to normal such injections have little influence on tetany. Moreover, the condition of hypoglycaemia precedes that of tetany. It is therefore suggested that the removal of the thyroids and parathyroids gives rise to two distinct effects, one being manifested upon the blood sugar regulating mechanism, causing hypoglycaemia, the other acting upon the nervous system, producing tetany.

2. Calcium appears to be intimately associated with both effects, for injections of calcium lactate will temporarily restore blood sugar to normal and also abolish tetany for a time.

3. Calcium may play an important rôle in maintaining the equilibrium of the blood sugar regulating mechanism during normal life.

THE COMPARATIVE EFFICIENCY FOR MILK PRODUCTION OF THE NITROGEN OF ALFALFA HAY AND THE CORN GRAIN.

PRELIMINARY OBSERVATIONS ON THE EFFECT OF DIURESIS ON MILK SECRETION.[1]

By E. B. HART and G. C. HUMPHREY.

With the Coöperation of J. J. Willaman and A. R. Lamb.

(From the Departments of Agricultural Chemistry and Animal Husbandry of the University of Wisconsin.)

(Received for publication August 3, 1914.)

In 1912 we published from this laboratory[2] the results of a study of the comparative efficiency for growth of the nitrogen of the corn kernel and alfalfa hay. From the data presented we were led to the conclusion that on the plane of nitrogen intake used in those experiments there was no appreciable differences in the utilization of the two sources of nitrogen for tissue building. The view is rather general that the roughages are rich in "amide" nitrogen, which term should include the amino-acid nitrogen and that in acid amides. These forms of nitrogen have in general been assumed to be of less nutritive value than the more complex proteins. With the newer developments in protein metabolism it is now positively established that direct use can be made of the amino-acids, but of course for an efficient use it is also recognized that in a mixture of these substances quantitative relations, as well as structure, will be important factors. Whether acid amide nitrogen can be utilized for protein synthesis is less clear, although a large amount of work has been done, especially with asparagine; but by the use of this body with its two forms of nitrogen linkage, it cannot be expected that clear results will be secured on the effect of the acid amide nitrogen.

[1] Published by permission of the Director of the Agricultural Experiment Station.

[2] This *Journal*, xiii, p. 133.

The amount of acid amide nitrogen in the coarse fodders is probably not large. Data are available on the amount of "amide" nitrogen in fodders secured by the use of Stutzer's reagent, or by tannic acid, but it is now known that the filtrate from these determinations may contain complex nitrogenous groups probably of polypeptide nature and of undoubted nutritive value, which would be classed as "amide" nitrogen in these methods of analysis. In a sample of alfalfa hay the nitrogen distribution as ammonia, acid amide and amino-acid nitrogen were as follows; the results are expressed as per cent of the total nitrogen in the hay:

	per cent
Nitrogen as ammonia	0.31
Nitrogen as acid amide	1.03
Nitrogen as amino-acid	10.17

The determination of ammonia was made by Folin's method, acid amide nitrogen by distillation with magnesium oxide at ordinary pressure and the amino-acid nitrogen by Van Slyke's method. From a consideration of the above data it is not surprising to find the nitrogen of alfalfa hay quite as efficient for growth as that from the corn kernel if such considerations were based upon the proportion of ammonia and acid amide nitrogen. The acid amide content is relatively small and the real nutritive differences in the nitrogen of the two materials would more probably rest upon the nature of the total amino-acid content derived both from complex proteins and preëxisting free amino-acids than upon the proportion of "amide" nitrogen.

Because of the very great use of alfalfa hay in milk production our studies have been extended to include observations on the efficiency of this nitrogen as compared with that of the corn kernel for such purposes.

The plan of the work involved the use of animals in full milk, with complete nitrogen balances, weights of milk, etc. A preliminary feeding period of ten days to two weeks was always included before beginning the quantitative collection of material. Records were secured usually for four weeks on the corn ration and then the same animal changed to the alfalfa ration, or vice versa for a like period. These changes were repeated a number of times for each animal, excluding in this way the effect of advancing lacta-

tion on the flow of milk. The changes were also made abrupt, but without apparent disturbance to the animals. No trouble of consumption of the ration was noticed, although usually the animals ate the alfalfa-starch ration with less avidity than the corn ration. After determining what amount of the ration would be entirely consumed, that proportion was continued through the several periods. Animal 1 was not with calf; animal 2 was pregnant. The alfalfa used was a good quality of western grown hay.

Experiment I.

Two grade Holsteins in full milk were used in this experiment. The composition of the rations was as follows:

TABLE I.

Composition of rations used.

	WEIGHT	N	TOTAL N	DIGESTIBLE N	FACTOR OF DIGESTIBILITY	PRODUCTION THERMS	N. R.
	pounds	*per cent*	*grams*	*grams*			
Ration 1							
Corn meal.....	8	1.44	52.2	35.5	68	7.1	
Gluten feed.....	4	4.15	75.7	64.1	85	3.1	
Corn stover.....	12	0.88	47.8	21.5	45	3.1	
Total.....	24		175.5	121.1		13.3	1 : 7.7
Ration 2							
Alfalfa hay.....	18	2.10	171.5	126.8	74	6.2	
Corn starch.....	7	0.07	2.2	2.2	100	7.0	
Total.....	25		173.7	129.0		13.2	1 : 7.7

The factors used for calculating the digestible nitrogen were taken from Henry's *Feeds and Feeding*. To secure a proper energy supply in the alfalfa ration corn starch was added. From the figures presented in the above table it will be seen that with a consumption of approximately equal quantities of air-dried matter the total therms and total nitrogen intake were approximately alike in the two rations. Each pound of the corn ration contained

7.31 grams of nitrogen, while each pound of the alfalfa ration contained 6.95 grams. In addition the digestible nitrogen in the two rations was closely comparable if we accept the approximate accuracy of the factors used. For the most part during any two periods of comparison the total air-dried matter consumed was kept alike which occasioned but slight variation in the total nitrogen intake. These variations in the nitrogen intake were too small, however, to account for the differences in milk secretion observed.

TABLE II.

Animal 1. Record of nitrogen balance, milk nitrogen, urine output, etc.

DATE	N INTAKE	FECES N	URINE	URINE N	MILK	MILK N	N BALANCE
	grams	grams	grams	grams	grams	grams	
Corn ration							
December 3-9	1147	459.6	76806	387.7	79758	364.7	−64.6
December 10-16	1052	404.9	71617	348.1	79022	339.4	−39.9
December 17-23	1023	372.8	55101	360.3	76298	364.8	−74.6
December 24-30	1023	394.3	69175	321.9	75702	347.8	−41.6
Alfalfa ration							
December 31-January 6	973	372.1	85432	328.18	70082	321.5	−48.8
January 7-13	973	335.3	89121	345.5	65127	288.4	+ 3.7
January 14-20	973	391.0	73231	356.34	59185	266.2	−40.6
January 21-27	973	382.6	73064	284.4	59838	281.1	+24.7
Corn ration							
January 28-February 3	1023	364.3	51098	336.16	64081	288.2	+34.4
February 4-10	1023	425.8	48438	315.5	71642	328.9	−46.9
February 11-17	1023	390.5	52719	318.1	69588	326.6	−11.9
February 18-24	1023	405.9	46660	270.7	70532	324.1	+22.7
Alfalfa ration							
Feburary 25-March 3	1021	423.2	80264	271.6	64828	320.6	+6.4
March 4-10	1021	424.6	70928	290.4	59701	298.4	+7.9

In table II are recorded the nitrogen balances, milk secretion and nitrogen elaboration in the milk by weekly periods. The figures represent the intake or outgo for the total seven days.

Animal 1 weighed 1053 pounds on December 3 and 1039 pounds on February 17, which indicates a maintenance of live weight.

The records for animal 2 which follow are much shorter owing to the inability to sustain this animal in milk flow. This cow was with calf, which may have had some influence in checking milk secretion, although she was not far along in the period of gestation. The records are of no value as indicating the comparative nutritive worth of corn and alfalfa nitrogen, but they do show the diuretic effect of alfalfa hay and for that reason are presented. This animal weighed 1068 pounds on December 3 and 1093 pounds on January 13.

TABLE III.

Animal 2. Record of nitrogen balance, milk nitrogen, urine output, etc.

DATE	N INTAKE	FECES N	URINE	URINE N	MILK	MILK N	N BALANCE
	grams	*grams*	*grams*	*grams*	*grams*	*grams*	
Alfalfa ration							
December 3–9	1000.7	460.3	70280	331.6	54220	270.1	−61.4
December 10–16	973.0	353.2	76414	423.7	48290	240.9	−44.9
December 17–23	973.0	355.0	98983	383.6	44685	223.4	+10.8
December 24–30	973.0	381.5	65826	289.6	38016	224.1	+77.6
Corn ration							
December 31–January 6	1023	443.4	44658	310.5	31946	195.4	+74.0
January 7–13	1023	469.8	52601	283.8	24370	184.8	+84.9

Experiment II.

For the records of the two animals that follow alfalfa of superior quality and second and third cuttings was procured from the Hoard Dairy Farm, Fort Atkinson, Wisconsin. Again two grade Holsteins in full milk and without calf were used for this work. The rations were of the following composition:

TABLE IV.

Composition of rations used.

	WEIGHT	N	TOTAL N	DIGESTIBLE N	FACTOR OF DIGESTIBILITY	PRODUCTION THERMS	N. R.
	pounds	*per cent*	*grams*	*grams*			
Ration 1							
Corn meal	6	1.59	43.7	29.7	68	5.3	
Gluten feed	6	4.12	112.7	95.2	85	4.6	
Corn stover	12	0.86	46.8	21.0	45	3.2	
Total	24		203.2	145.9		13.1	1:6.4
Ration 2							
Alfalfa hay	16.0	2.81	203.2	150.2	74	5.5	
Corn starch	7.5	0.10	3.6	3.6	100	7.5	
Total	23.5		206.8	153.8		13.0	1:6.8

Because of the higher nitrogen content of the alfalfa hay used it was possible in this experiment to keep the nitrogen intake at a higher level than in the previous experiment. Every pound of the corn ration contained 8.46 grams of nitrogen, while a pound of alfalfa ration contained 8.80 grams of nitrogen. The two rations were in close agreement in reference to total nitrogen, digestible nitrogen and production therms. The records of the animals are displayed in the tables that follow. Animal No. 3 weighed 1149 pounds when the experiment was initiated and 1145 pounds at its termination.

It should be observed that in the alfalfa period an increase of two pounds per day was made in the daily ration which explains the increase in intake of nitrogen. This was not done, however, until after a record of two weeks on the lower plane of nitrogen had been secured.

Animal 4 weighed 862 pounds when the experiment was started and 825 pounds at its close.

As in the case of animal 3 there was an increase in the daily ration of 2 pounds during the corn period which accounts for the increase in nitrogen consumed.

TABLE V.

Animal 3. Record of nitrogen balance, milk nitrogen, urine output, etc.

DATE	N INTAKE	FECES N	URINE	URINE N	MILK	MILK N	N BALANCE
	grams	*grams*	*grams*	*grams*	*grams*	*grams*	
Corn Ration.							
December 6–12	1302	449.3	28292	467.1	74853	414.6	− 29.3
December 13–19	1302	417.3	27024	437 2	72319	375.6	+ 72.4
December 20–26	1302	436.5	25210	350.2	70911	382.5	+133.4
December 27–January 2	1302	434.2	22710	433.3	70095	422.4	+ 12.8
Alfalfa Ration							
January 3–9	1355	408.1	41642	491.7	61741	351.6	+104.0
January 10–16	1355	507.3	64548	708.4	55390	298.7	−159.0
January 17–23	1478	533.6	38523	525.3	54292	309.1	+110.4
January 24–30	1478	487.3	47793	607.8	61505	329.2	+ 54.0
Corn Ration							
January 31–February 6	1421	434.3	28771	557.2	62366	286.6	+143.1
February 7–13	1421	413.0	35917	330.1	68672	349.0	+329.1

TABLE VI.

Animal 4. Record of nitrogen balance, milk nitrogen, urine, output, etc.

DATE	N INTAKE	FECES N	URINE	URINE N	MILK	MILK N	N BALANCE
	grams	*grams*	*grams*	*grams*	*grams*	*grams*	
Alfalfa Ration							
December 6–12	1108	360.4	32730	521.1	76532	421.0	−194.3
December 13–19	1108	554.8	52276	492.4	76487	366.7	−305.2
December 20–26	1108	408.2	42820	514.2	90705	383.4	−197.0
December 27–January 2	1108	397.3	41918	488.2	84476	362.6	−140.0
Corn Ration							
January 3–9	1066	430.3	36141	380.4	82151	352.3	− 97.0
January 10–16	1066	409.0	57943	446.5	79381	317.1	−106.5
January 17–23	1184	385.2	71249	359.6	82496	329.8	+109.7
January 24–30	1184	428.3	80454	317.0	82054	335.0	+104.1
Alfalfa Ration							
January 31–February 6	1232	412.2	82381	414.6	81508	357.6	+ 47.6
February 7–13	1232	437.4	79886	516.4	70713	317.6	− 39.6

From a review of the data there are two general deductions that may be made: 1. A general trend toward the elaboration of as much nitrogen-containing material in the milk on the alfalfa ration as on the corn ration; 2. A fall in the flow of the milk as we pass from the corn to the alfalfa ration with an accompanying rise in the urinary excretion; the converse of this is also true. The daily difference in milk flow between a corn and alfalfa period was variable, but reached in the extreme points of the curve 5 to 6 pounds on a total secretion of 20 to 25 pounds.

TABLE VII.

Comparison of grams of nitrogen secreted in milk during four week periods.

	CORN N	ALFALFA N	CORN N	ALFALFA N
	grams	*grams*	*grams*	*grams*
Animal 1. December 3–April 10...	1414	1156	1266	1232
Animal 3. December 6–February 13.	1593	1287	1270	

	ALFALFA N	CORN N	ALFALFA N
	grams	*grams*	*grams*
Animal 4. December 6–February 13.	1532	1334	1348

The facts tending to support the first statement are summarized in table VII. Here are brought together the grams of nitrogen secreted during like periods (four weeks) on the two rations by three of the animals. Animal 2 is not included because of the constant tendency to lower the milk secretion at a rapid rate as lactation advanced. In the last period given for animals 3 and 4, the nitrogen secretion observed, which was for but two weeks, is merely doubled for purposes of comparison.

There was in general a larger milk flow and a greater secretion of milk proteins during the first periods of observation than during the second, after which the variations were less marked. While there was a fall in the milk proteins produced by animals

1 and 3 when changed from corn to alfalfa the same fact is observed with animal 4 in the change from alfalfa to corn. Again the failure of a rise in milk nitrogen production in the later periods on corn as compared with the previous alfalfa period, or the sustained nitrogen production on alfalfa as compared with the previous corn period illustrates well the fallacy that might arise should judgment of the efficiency of these sources of nitrogen be placed on the records for the first periods alone. It is apparent from all the data that the nitrogen of alfalfa hay is probably quite as effective for milk protein building as the more complex proteins of the corn kernel.

Had there been a consistent fall in the total nitrogen secretion in the milk during the alfalfa feeding it should have been possible to increase this secretion by the addition to the ration of complex proteins. To test this theory 0.3 pound of wheat gluten was added to the alfalfa ration during a period of two weeks. This was done with both animals 3 and 4 and begun on March 21. The 0.3 pound of wheat gluten was equivalent to approximately 0.020 gram of nitrogen or about one-tenth of the total nitrogen intake of the animal and would be approximately equivalent to the "amide" nitrogen of the alfalfa hay. During the period of two weeks immediately preceding this observation the amount of milk nitrogen elaborated by animal 3 on the alfalfa ration was 580 grams in 203 pounds of milk. During the next two weeks on the alfalfa ration, plus wheat gluten, the amount produced was 566 grams in 195 pounds of milk. Animal 4 produced in the two weeks' period, on the corn ration immediately preceding the alfalfa plus wheat gluten ration, 629 grams of nitrogen in 308 pounds of milk and 649 grams of nitrogen in 286 pounds of milk on the alfalfa-wheat gluten ration.

These data are decidedly confirmatory of the larger body of data presented namely, that the alfalfa nitrogen served the purpose of milk protein building quite as effectively as that of the corn kernel. In addition, the amount of nitrogen absorbed from the tract on the two rations was very closely alike, approximating 60 to 65 per cent of the total nitrogen ingested.

What influence, if any, the alfalfa may have had in lowering the elaboration of milk protein must be considered in the general tendency of this feed to lower the volume and weight of milk

produced. This phenomenon of a lowered weight of milk during an alfalfa period when compared with the preceding or following corn period was very general. Animal 1 secreted 684 pounds of milk during the first corn period, 560 pounds during the alfalfa period of like duration and 607 pounds during the second corn period. Animal 3 secreted 635 pounds of milk during the corn period and 510 pounds during the alfalfa period. Animal 4 was the interesting exception to this record, secreting 723 pounds during the alfalfa period and 718 pounds during the following corn period. With animal 4, however, it should be observed that the secretion of urine was not stimulated to as great a volume during the alfalfa period as compared with the corn period.

These facts suggest a close relation between the diuretic effect of the alfalfa hay with certain animals and the fall in milk flow and is very probably the factor in the explanation of our results. In the following charts are shown these relations of the flow of urine to milk production.

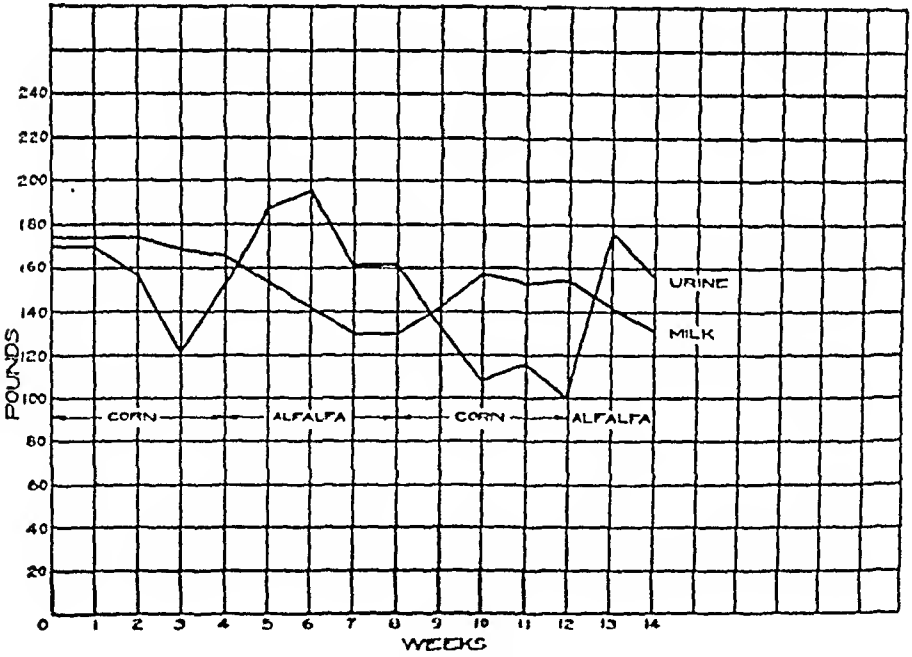


Chart 1. Animal 1. Showing the diuretic effect of alfalfa and its effect on milk secretion.

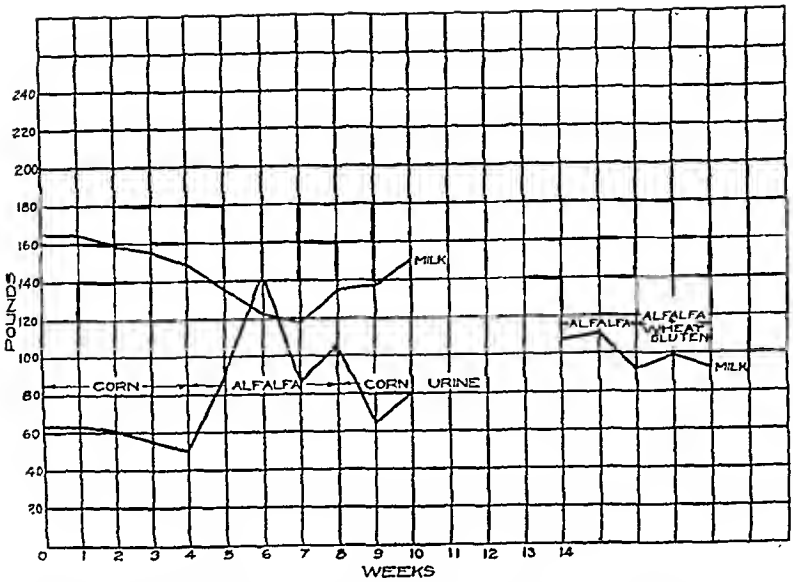


CHART 2. Animal 3. Showing the diuretic effect of alfalfa and its effect on milk secretion.

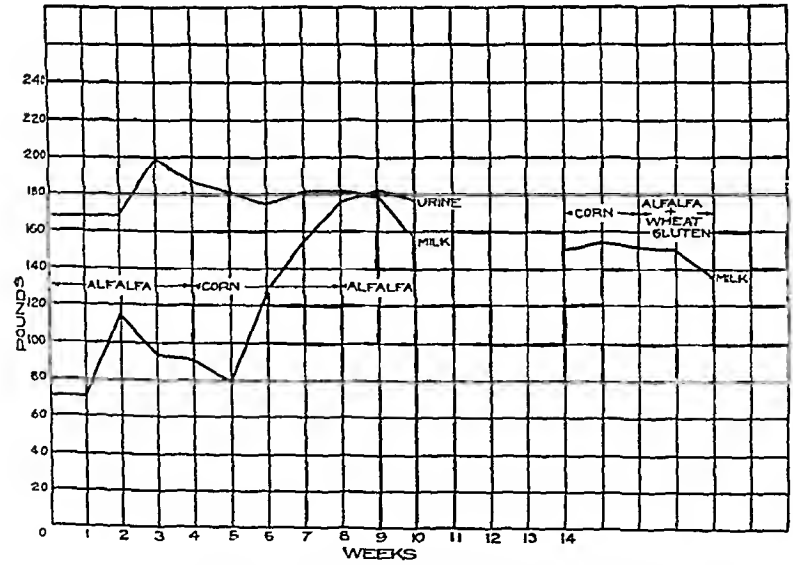


CHART 3. Animal 4. Diuretic effect of alfalfa. In this case the diuretic effect was not apparent until the second period of alfalfa feeding.

When the flow of milk was depressed, as during an alfalfa period, the percentage of nitrogen in the milk was usually slightly higher, which would tend to equalize the total quantity of protein elaborated during any two periods. For example, during a corn period (high flow) animal 1 averaged 0.45 per cent of nitrogen in the milk, while during the alfalfa period (low flow), this was 0.49 per cent. With animal 3 during the alfalfa period the average percentage of nitrogen was 0.56, while it was 0.48 during the corn period. That we may have an increase in solids in milk accompanied by a decreased flow is a well known fact evidenced by what takes place during a normal lactation period. A good illustration of this was observed with animal 2 which was with calf and constantly and rapidly decreasing her flow of milk as lactation advanced. When her flow of milk was 16 to 20 pounds the percentage of nitrogen was approximately 0.49, but when the flow had decreased to 3 to 5 pounds the percentage of nitrogen had risen to 0.91. While of course the secretion of nitrogen does not keep pace sufficiently fast in such an extreme case to equal the total nitrogen elaboration during the higher flow, yet there is a tendency in that direction. Where the differences in flow were much less, as in the observations recorded with non-pregnant animals and incident to the diuretic effect of alfalfa, the compensation for a lower secretion by the higher percentage of nitrogen became more perfect.

To what the diuretic effect of the alfalfa was due is not wholly clear. The mineral content of the two rations is not so widely different except in the amount of calcium and silicon. In table VIII is given the mineral content of the two rations for comparison.

TABLE VIII.

Mineral content of corn and alfalfa ration.

	Lbs.	CaO	MgO	K_2O	Na_2O	P_2O_5	SO_3	Cl	SiO_2
		grams	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*
Corn meal	8	1 23	7.50	18 8	1 08	23.60	12.76	1.60	1.75
Gluten feed	4	7 60	9 46	6 32	0 52	21 50	25 21	3.01	0.92
Corn stover	12	40 20	39 60	112.53	6 50	27 21	15.65	4.51	256 9
		49 03	56 56	137 65	8 10	73 31	53.65	9.15	259 56
Alfalfa	18	319.5	40 86	145 2	15 1	49 4	59.02	21 5	84.72
Starch	7								

The corn ration, being much higher in silicon, may contain the bases in a much more insoluble form as salts of silicic acid and consequently lessen any diuretic effect they may have. It is, of course, well known that certain salts are excellent diuretics, but so far as we are aware their effect on milk flow has not been studied. It is also possible that alfalfa may contain some organic substance with specific diuretic effect. These are matters calling for further investigation.

In addition, one might expect that where water was given *ad libitum* as was done in these experiments there would be a sufficient amount drunk to compensate for the increased flow of urine, with a maintenance of the milk flow. Our records on water consumption were rather short. During an alfalfa period when data on water consumption were collected on animal 4, but a slight increase in daily consumption was observed as compared with the corn period. During the alfalfa period of two weeks there was an average daily consumption of 71 pounds, while 68 pounds were drunk daily during the corn period. During a like period animal 3 consumed daily 78 pounds of water on the alfalfa ration and 68 pounds daily on the corn period. Apparently there was no appreciable withdrawal of water from the tissues, but a slight stimulation to the thirst center when diuresis occurred. The constant stimulation to the renal cells induced by the diuretic substance of alfalfa apparently occasioned a somewhat different partition of the water drunk than in the case of the corn ration. Even if generally more water was drunk during the alfalfa period, as is very probable, it is apparent that the animals were not able under the constant stimulation of the renal cells to maintain the flow of milk at the same level as where no diuretic substances were present.

Whether a longer period of observation than four weeks of alfalfa feeding would have brought the flow of milk back to that of the corn period remains for further observations. What practical bearing this experiment may have cannot be forecasted until a larger number of animals have been involved with longer periods of observation on mixed rations. Such observations will be made as soon as possible, together with more detailed studies on the specific effects of diuretics on milk secretion.

SUMMARY.

Data are presented on the comparative value for milk protein production of the nitrogen from alfalfa hay and the corn kernel. The data indicate that on the plane of intake used the nitrogen of alfalfa hay is as effective for milk protein building as that of the corn kernel.

The acid amide nitrogen of alfalfa is very low in amount, constituting about 1 per cent of the total nitrogen, while the amino acid nitrogen makes about 10 per cent of the total nitrogen. It is well established that amino nitrogen has nutritive value and that of alfalfa hay is probably not an exception. Our experiments give no indication of the value of the acid amide nitrogen.

The real nutritive value of the nitrogen of roughages should rest upon the nature of the total amino acid content derived from more complex proteins and preëxisting free amino acids rather than upon the proportion of "amide" nitrogen as found by the Stutzer method.

Alfalfa hay has specific diuretic properties and its ingestion was generally followed by a marked rise in the output of urine. This rise in renal activity caused a depression in the milk flow which again rose in volume as the alfalfa hay was withdrawn from the ration. The diuretic stimulus caused in some cases a shrinkage in volume of 5 to 6 pounds on a flow of 25 pounds daily. It is possible that this diuretic effect is due to salts contained in the hay, yet the possibility of the presence of specific substances of organic nature is not excluded.

THE MODE OF ACTION OF UREASE AND OF ENZYMES IN GENERAL.

By DONALD D. VAN SLYKE and GLENN E. CULLEN.

(*From the Rockefeller Institute for Medical Research, New York.*)

(Received for publication, July 14, 1914.)

The fact that the soy bean contains a very active enzyme which splits urea into ammonium carbonate was discovered by Takeuchi[1] who in establishing the specificity of the enzyme demonstrated that it does not attack alanine, allantoin, arginine, benzamide, glycine, guanine, leucine, tyrosine, histidine, creatinine, uric acid, hippuric acid and biuret.

The Armstrongs with Horton and Benjamin have recently published three papers[2] on the mode of action of the urease. In confirming its specificity they have shown that it is without action even on derivatives of urea, such as methylurea, *s*-dimethylurea *as*-dimethylurea, ethylurea, *s*-diethylurea.

They have also plotted the time curve of the reaction, showing that the rate decreases as the reaction proceeds, and have demonstrated that the ammonium carbonate produced by the reaction has an appreciably retarding influence on it. They have furthermore studied the influence of various mineral and organic substances on the rate of the urease action, the most striking results being, perhaps, those with carbon dioxide, which greatly accelerates the reaction. Mineral acids and alkalies retard or altogether stop the reaction.

The two products, ammonia and carbon dioxide, show opposite effects, ammonia greatly retarding the reaction, while carbon dioxide accelerates it to an equally striking degree. Hydrocyanic acid and glycocoll accelerate like carbonic acid. Ammonium

[1] Takeuchi: *Journ. of the Coll. of Agriculture*, Tokyo, Part 1. p. 1, 1909.

[2] H. E. Armstrong and Horton: *Proc. Royal Soc.*, lxxxv, p. 109, 1912; H. E. Armstrong, Benjamin and Horton: *ibid.*, lxxxvi, p. 328, 1913; E. F. and H. E. Armstrong: *ibid.*, lxxxvi, p. 561, 1913.

carbonate, though it retards less than ammonia, has a depressing effect on the enzyme, so that the action of the latter on urea is self-retarding. The neutral salts, sodium and potassium chloride, in sufficient concentration retard the action somewhat. Doubling the enzyme concentration approximately doubled the rate of ammonia formation. With constant enzyme, the rate is not, however, proportional to the urea concentration. Consequently the reaction does not follow the law of mass action in its ordinary form. The conclusion is drawn that "enzymic action takes place entirely at the surfaces of colloid particles suspended in the solution of the hydrolyte and not between substances which are all in true solution."[3] This hypothesis is developed more fully by E. F. and H. E. Armstrong in a later paper.[4] As will be seen later, we have succeeded in explaining the facts by a simple application of the law of mass action. In regard to the facts themselves, however, our results accord with those of the above mentioned authors at every point on which we have touched.

That the rule—reaction rate proportional to enzyme concentration—holds, not only when the enzyme is doubled, but over as wide a range of concentrations as it is practicable to work with, has been recently shown independently by Marshall[5] and by ourselves.[6]

The original object of our work was to determine whether the enzyme belongs to the class of contact catalysts, or acts by the formation of an intermediate labile compound with the substrate. If it acts by mere contact, the reaction velocity, enzyme concentration being kept constant, should increase in direct proportion to the urea concentration, as the number of contacts per time unit between each enzyme particle and the surrounding urea molecules would be proportional to the number of the latter. If the enzyme acts by formation of an intermediate compound with the urea, however, one would expect conditions to be different. So long as sufficient urea were present to keep all the enzyme combined and therefore acting, the presence of excess urea be-

[3] *Proc. Royal Soc.*, lxxxvi, p. 343, 1913.

[4] *Ibid.*, lxxxvi, p. 561, 1913.

[5] Marshall: This *Journal*, xvii, p. 351, 1914.

[6] Van Slyke and Cullen: Report to Am. Soc. Biol. Chem., December, 1913; Proceedings, this *Journal*, March, 1914, p. xxviii.

yond this point would not accelerate the reaction. The excess urea would merely remain inert, awaiting its opportunity to combine with enzyme. The following experiment shows that this is actually the case.

Experiment 1. Effect of concentration of urea, enzyme concentration being constant.

One-cc. portions of *fresh* enzyme solution (made by extracting 1 part of soy bean meal with 5 parts of water) were mixed at 20° with 5-cc. portions of urea solutions of varying concentration. Enzyme and urea were mixed in 100 cc. tubes, which were closed and kept at 20° for exactly fifteen minutes.

TABLE 1.

UREA CONCENTRATION	$\frac{N}{50}$ HCl NEUTRALIZED BY AMMONIA FREED IN 15 MINUTES	TOTAL UREA DECOMPOSED
per cent	*cc.*	*per cent*
0.2	16.56	99.7
0.4	25.56	77.0
0.6	25.62	51.5
0.8	25.18	38.0
1.0	25.40	30.6
2.0	25.86	15.5
3.0	25.88	9.6
4.0	25.17	7.6
5.0	25.47	6.1
10.0	25.04	3.0
20.0	19.30	1.1
40.0	12.00	0.4

The action of the enzyme was then checked by addition of 2 cc. of saturated potassium carbonate solution, and the ammonia driven into $\frac{N}{50}$ HCl by aeration.

In the 0.2 per cent solution all the urea was decomposed, yielding 16.56 cc. of $\frac{N}{50}$ NH_3 (calculated, 16.6). In the 0.4 percent solution, however, only 25.56 out of a possible 33.2 cc. of $\frac{N}{50}$ NH_3 were found. In concentrations from 0.4 to 10 per cent, the rate of the reaction remained the same. Between 10 and 20 per cent, the excess urea begins to exert a depressing effect on the reaction, probably due to the physical effect of solutions of such high concentration.

The conclusion which one might draw from the results with urea solutions up to 10 per cent is, that a given amount of enzyme can, under given conditions, decompose a definite amount of

urea in unit time, the presence of more urea than this amount having no influence on the progress of the reaction.

The following experiment shows, however, that when the urea concentration falls below a certain limit, the rate of ammonia formation also falls. A definite, though rather low concentration of excess urea is necessary to keep the enzyme working at its maximum rate.

Experiment 2. Effect of decreasing urea concentration on reaction as latter approaches completion.

The technique was the same as in Experiment 1. Two series of determinations were run, all conditions being identical in both, except the urea concentration. This was so chosen that in Series 1 the urea was completely decomposed at the end of the experimental period, while in Series 2 so much urea was added that three-fourths of it remained unattacked at the close of the period.

TABLE II.

TIME OF REACTION	SERIES 1		SERIES 2	
	1 cc. enzyme + 5 cc. 1 per cent urea		1 cc. enzyme + 5 cc. 5 per cent urea	
	$\frac{N}{50}$ NH_3 formed	Total urea decomposed	$\frac{N}{50}$ NH_3 formed	Total urea decomposed
minutes	*cc.*	*per cent*	*cc.*	*per cent*
20	25.9	32.0	26.0	6.3
40	41.3	49.8	40 1	9.6
60	53.1	64.6	53.2	12.8
80	64.3	77.5	65.7	15.3
100	75.3	90.7	76.0	18.3
120	79.6	95.9	87.1	21.0
140	81.6	98.3	96 0	23.1
160	83.1	100.0	105 8	25 5

The curves of figure 1, afford a comparison of the two series. The reaction rate in both remains practically the same until 90 per cent of the urea in Series 1 has been destroyed. From this point the rate becomes rapidly slower as the urea is still further decreased. In the other series, where the greater supply of urea still leaves a large excess undecomposed, the rate continues on from this point practically undiminished to the end of the experiment. At the point where the two curves separate the concentration of the urea remaining in Series 1 is 0.013 molecular (0.08 per cent). It appears that in solutions containing only pure urea and enzyme, the

urea concentration cannot be reduced below this limit, without also reducing the rate of ammonia formation by the enzyme.

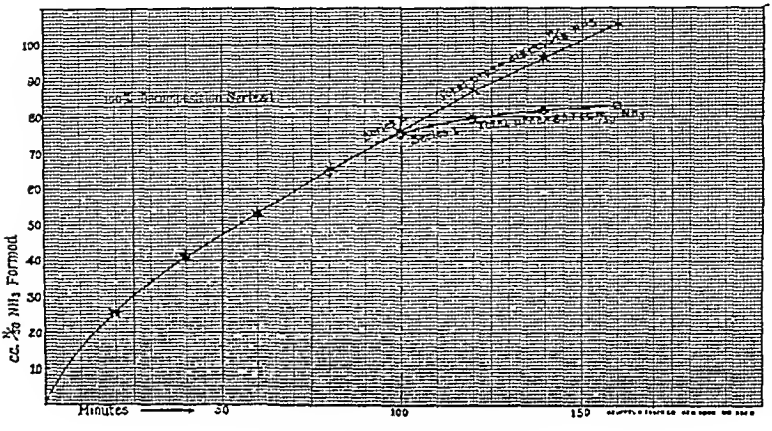


FIG. 1. From experiment 2.

As is demonstrated in the succeeding paper, the hydrogen ion concentration is a factor towards which urease is very sensitive. The alkalinity of the ammonium carbonate formed from the urea is sufficient to reduce the activity of the enzyme to a fraction of its value in neutral solution. In order to rule out this disturbing factor of changing reaction in a closer study of the conditions, we have used the well-known regulating mixtures of primary and secondary phosphates, choosing concentrations which were found (see succeeding paper with Zacharias) to keep the H′ concentration so near the optimum that it was constant so far as effect on the enzyme was concerned. Another effect of the phosphates, which was not foreseen, is a magnification of the influence of urea concentration on the reaction rate, the effect being to raise the minimum urea concentration causing maximum enzyme activity from the point 0.013 molecular, observed in the absence of salts, to a much higher figure. This behavior greatly broadened the zone of urea concentration over which effects could be observed, and was consequently of decided assistance in determining quantitatively the nature of the effect of changing urea concentration on the enzyme action.

Experiment 3. Effect of urea concentration on enzyme action in solutions kept neutral by phosphate mixture.

The phosphate solution used was made by mixing 2 volumes of $\frac{M}{1}$ K_2HPO_4 with 1 volume of $\frac{M}{1}$ KH_2PO_4. In each of a series of 100 cc. test tubes were placed 5 cc. of this mixture, 4 cc. of urea solution of varying concentration, and 1 cc. of a 1 per cent solution of precipitated urease (preparation described in the second succeeding paper). Each solution contained, therefore:

K_2HPO_4, $\frac{M}{3}$ concentration. } Total phosphate conc., $\frac{M}{2}$.
KH_2PO_4, $\frac{M}{6}$ concentration. }
Urease, 0.1 per cent.
Urea, varying.
Volume of each solution, 10 cc.

The reaction in each case ran sixty minutes at 20°.

TABLE III.

CONCENTRATION UREA	a 0.01 N NH_3 CALC. FOR COMPLETE DECOMP. OF UREA	x 0.01 N NH_3 FORMED	$0.4343\ c = \frac{d}{dEt-x} \log \frac{a}{a-x}$	d	$K = \frac{1}{t} \log \frac{a}{a-x}$
per cent	*cc.*	*cc.*			
0.0375	12.5	5.8	0.055		0.0045
0.075	25.0	10.4	0.058		0.0039
0.15	50.0	15.5	0.053		0.0027
0.3	100.0	21.2	0.052		0.0016
0.6	200.0	24.8	0.051		0.0009
1.2	400.0	27.0	0.052		0.0005
2.4	800.0	28.5	0.052		0.0004
4.8	1600.0	31.0		5.17	0.0002
9.6	3200.0	31.0		5.17	0.0001
Average.....			0.053		

Formulation of the nature and course of the reaction.

The above three experiments support the following explanation: The process consists of two reactions, (1) enzyme and urea combine: neglecting the part played by water, we may express this reaction by the equation, $E + U = [EU]$. (2) The combination splits $[EU] = E + CO_2 + 2NH_3$. The velocity of the first reaction is, according to the law of mass action, proportional to the product of enzyme and urea concentrations. With a sufficient concentration of excess urea, this reaction is so rapid that it con-

sumes, compared with the second reaction, an insignificant time interval. The pace is set entirely by the slower decomposition reaction, $[EU] = E + CO_2 + 2NH_3$, of which the velocity de-

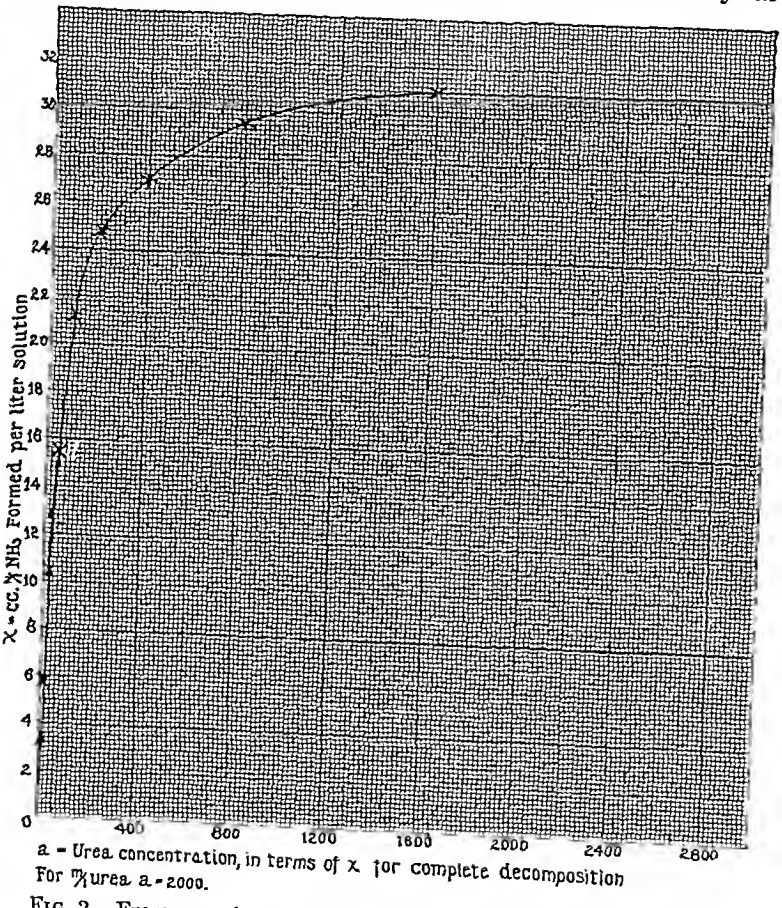


FIG. 2. From experiment 3. Effect of urea concentration on amount of urea decomposed in one hour. Solutions kept neutral by half-molecular phosphate mixture. Enzyme concentration, 0.1 per cent.

pends only on the amount of reacting enzyme present (condition of Experiment 1 and two highest concentrations of Experiment 3).

As, in the course of the reaction, the urea concentration falls, however, the rate at which the enzyme-urea combination is formed

falls in proportion, and ultimately become so slow that the first stage does add an appreciable fraction to the total reaction time. The result is, that from this point the rate of ammonia formation falls continuously and appreciably with the urea concentration (last stage of Series 1, Experiment 2, figure 1).

The above explanation may be formulated as follows:

Let Θ represent the time required for an enzyme molecule to complete the cycle of the reaction, *i.e.*, to combine with a urea molecule, and to throw it off as ammonia and carbon dioxide. The total time Θ, is the sum of the intervals consumed by the two separate stages, and may be expressed as such in the following manner:

(1) $$\Theta = \frac{1}{cU} + \frac{1}{d}$$

c is the velocity constant of the first reaction $E + U = [EU]$. This reaction being directly proportional to the urea concentration U, the time required for it is $\frac{1}{cU}$.

d is the velocity constant of the second reaction, $[EU] = E + CO_2 + NH_3$. The time required for this reaction is independent of the urea concentration, and, being inversely proportional to the velocity, is expressed by $\frac{1}{d}$.

The velocity, $\frac{dx}{dt}$ of the total process, *i.e.*, of ammonia formation, is inversely proportional to the time Θ required for the successive consummation of the two stages.

(2) $$\frac{dx}{dt} = \frac{1}{\Theta} = \frac{1}{\frac{1}{cU} + \frac{1}{d}} = \frac{c\,d\,U}{d + c\,U}$$

In order to express the equation in terms of x, we substitute $a - x$ for U, a representing the initial urea concentration, x the amount transformed into ammonia after t time units. Thus:

(3) $$\frac{dx}{dt} = \frac{c\,d\,(a-x)}{d + c\,(a-x)}.$$

This expresses the velocity for unit enzyme concentration. As the amount of ammonia produced in unit time must be proportional to the number of enzyme molecules going through the

above formulated action, the velocity for variable enzyme concentration, is proportional to that concentration, which we represent by E. We then have:

$$\frac{dx}{dt} = E\,\frac{c\,d\,(a-x)}{d+c\,(a-x)}. \tag{4}$$

From this:

$$dx\left(\frac{1}{c\,(a-x)}+\frac{1}{d}\right) = Edt. \tag{5}$$

Integrating, we have:

$$-\frac{1}{c}\,ln\,(a-x)+\frac{x}{d} = E\,t+\text{const.} \tag{6}$$

Placing x and t equal to zero, we find:

$$\text{const.} = -\frac{1}{c}\log a$$

Substituting this value for the constant in (6), we have:

$$t = \frac{1}{E}\left(\frac{1}{c}\,ln\,\frac{a}{a-x}+\frac{x}{d}\right) \tag{7}$$

The constant d is readily and accurately found from the values of x and t determined when the urea concentration is so high, that the rate is independent of it, the term $\frac{1}{c}\,ln\,\frac{a}{a-x}$ becoming negligible (see highest concentrations of Experiment 3, for example). Then:

$$d = \frac{x}{Et} \tag{8}$$

d having been determined, c is found from values of x and t determined when the concentration of excess urea is so low that it does affect the reaction rate. From (7) we have:

$$c = \frac{d}{d\,tE-x}.\quad ln\frac{a}{a-x} \text{ or } 0.4343\,c = \frac{d}{d\,tE-x}\log_{10}\frac{a}{a-x} \tag{9}$$

In applying the above considerations to the results of Experiment 3, we take 1 per cent as unit enzyme concentration. d is determined from the value attained by x in urea concentrations

so high (4.8 per cent or over) that the reaction rate is at its maximum.

$$d = \frac{x}{Et} = \frac{31}{0.1 \times 60} = 5.17$$

By substituting this value for d into equation (9), we then calculate the value of c from results over the range where c has appreciable effect, *i.e.*, where x has not yet reached its maximum. As one sees from the next to last column of the table, c, determined over a great range of urea concentrations, proves to be a genuine constant, its variations being no greater than one would expect from the experimental error attending velocity measurements.

For comparison the values of the ordinary first order constant $K = \frac{1}{t}\log\frac{a}{a-x}$ are given in the last column. It is evident that this equation does not hold as even a distant approximation.

The following series demonstrate that the equation $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$ holds, not only for the initial rate of reaction with constant enzyme concentration, as in the Experiment 3, but also for the entire course from the start to the time of complete decomposition of the substrate, and for varying enzyme concentrations.

Experiments 4, 5 and 6. Complete course of reaction in neutral phosphate solution with different enzyme concentrations.

Conditions are the same as in Experiment 3, except that the ammonia is determined, not for one time interval only, but at different intervals from the beginning of the reaction till it is nearly complete. The enzyme concentration is different in each experiment tabulated below. The urea is constant at 0.12 per cent (0.02 mol.), so that complete decomposition of 10 cc. of solution yields 40 cc. of $\frac{N}{100}$ NH_3, giving a the value 40. In order to facilitate the manipulation, 200 cc. of solution containing $\frac{M}{3}$ K_2HPO_4, $\frac{M}{3}$ KH_2PO_4, 0.12 per cent urea, and enzyme in the concentration used, were made up at once. The enzyme solution was added last, the other contents having been brought to 20° and to such a volume that the enzyme solution made the total up to the 200 cc. mark. Portions of 10 cc. were pipetted immediately into 100 cc. test tubes in the 20° bath. After adding 2 drops of octyl alcohol to each, these were closed with stoppers bearing the aerating tubes. A minute before the end of each succeeding interval one of the test tubes was removed from the bath and aerated into 25 cc. of $\frac{N}{50}$ HCl for a half minute

in order to recover any ammonia which might be free in the air space of the tube. At the exact end of the interval 6 grams of potassium carbonate were added to stop the enzyme action and free the ammonia. The aeration was then continued until all the ammonia had been driven over. Although the titrations were performed with $\frac{N}{50}$ solutions, the results for x are given in terms of $\frac{N}{100}$ NH_3 for each 10 cc. solution, as it is desirable to express results for these and subsequent experiments in values of x referring to the same unit, and the unit taken (cc. of $\frac{N}{100}$ NH_3 formed in 10 cc. of reacting solution = cc. $\frac{N}{1}$ NH_3 formed per liter) appears to be the most convenient.

Determination of d. Experiment 3 shows that in urea solutions of 4.8 to 9.6 per cent concentration the term $\frac{1}{c}\log\frac{a}{a-x}$ becomes negligible, so that the equation $d=\frac{x}{Et}$ holds. Consequently we have used 6 per cent urea solutions to determine d. The technique was the same described in the preceding paragraph. The precaution was taken, however, to keep the time intervals here short enough so that x did not exceed 40, in order that the neutrality of the solution might not be appreciably disturbed.

TABLE IV.

Experiment 4. Course of reaction with 0.03 per cent enzyme.

DETERMINATION OF d. $a = 2000$.			DETERMINATION OF c. $a = 40$.			
t	x	$d=\frac{x}{0.3\,t}$	t	x	$a-x$	$0.4343\,c=\frac{d}{dEt-x}\log\frac{a}{a-x}$
133	18.9	4.73	60	4.20	35.80	0.0527
133.5	19.3	4.80	120	8.40	31.60	0.0557
208	29.4	4.67	186	12.10	27.90	0.0520
Average.....		4.73	240	14.84	25.16	0.0500
			300	18.08	21.92	0.0503
			360	21.10	18.90	0.0513
			420	23.70	16.30	0.0513
			Average.....			$c=0.0519$

TABLE V.

Experiment 5. Course of reaction with 0.10 per cent enzyme. d determined in Experiment 8=5.17

DETERMINATION OF c. $a = 40$.

t	x	$a-x$	$0.4343\,c$
15	3.80	36.20	0.0581
30	7.24	32.76	0.0576
45	10.52	29.48	0.0549
60	13.28	26.72	0.0516
75	16.30	23.70	0.0526
90	18.90	21.10	0.0504
105	21.50	18.50	0.0517
120	23.40	16.60	0.0516
135	25.36	14.64	0.0510
150	27.34	12.46	0.0527
165	29.58	10.42	0.0548
180	31.30	8.70	0.0527
195	32.80	7.20	0.0570
225	34.54	5.46	0.0550
255	36.34	3.66	0.0576
285	37.40	2.60	0.0563
345	38.76	1.24	0.0562
375	38.94	1.06	0.0527
Average			$c=0.0541$

TABLE VI.

Experiment 6. Course of reaction with 0.80 per cent enzyme.

DETERMINATION OF d. $a = 2000$.

t	x	$d = \frac{x}{0.3\,t}$
20	31.20	5.20
25	38 00	5.07
28	43.10	5.15
28	42 48	5.06
Average		$d=5$ 12

DETERMINATION OF c. $a = 40$.

t	x	$a-x$	$0.4343\,c$
20	14 08	25 92	0.0577
30	20 22	19 78	0.0603
40	25.06	14.94	0.0600
51	29 20	10.80	0.0601
60	31.98	8 02	0.0593
75	35 12	4.88	0.0586
90	36 90	3 10	0.0559
105	38.00	2 00	0.0539
120	39 00	1.00	0.0563
135	39 54	0.46	0.0577
Average			$c=0.0579$

TABLE VII.

Summary of results of Experiments 3, 4, 5, and 6.

EXP. NO.	E PER CENT ENZYME	0.4343 c			d AVERAGE
		Maximum	Minimum	Average	
4	0.03	0.055	0.050	0.051	4.73
3	0.10	0.058	0.051	0.053	5.17
5	0.10	0.058	0.050	0.054	5.17
6	0.30	0.060	0.054	0.057	5.12

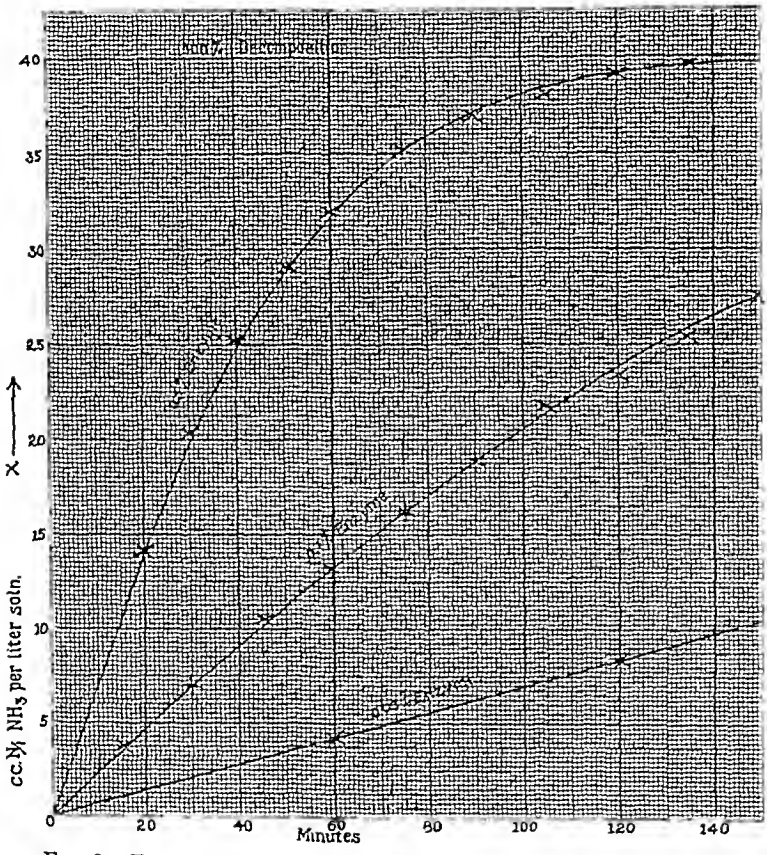


FIG. 3. From experiments 4, 5 and 6. Action of urease in solution kept neutral with phosphate. Curves are plotted from values of t calculated by the equation $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$. Crosses represent experimental results up to 150 minutes.

Some interesting results are obtained by dissecting the reaction with the aid of equation (7), $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x}+\frac{x}{d}\right)$. Here the term $\frac{1}{c}\log\frac{a}{a-x}$ represents, at any period of the reaction, that portion of the total time t which has been consumed in the formation of enzyme-urea complexes, while the term $\frac{x}{d}$ represents the time consumed in their disruption. In the history of a single urea molecule, the "combination interval" is the time intervening from the moment the enzyme molecule was freed from its last urea molecule until it unites with the next; the "disruption interval" is the period during which the enzyme and urea molecules remain combined, before the urea is split off as ammonium carbonate.

In the following table the values of the two terms of the equation are calculated separately from the results of Experiment 6, using the constants $c = 0.0577$ and $d = 5.12$. $E = 0.3$ per cent enzyme concentration.

TABLE VIII.

Analysis of results of Experiment 6.

x	$\frac{1}{Ec}\log\frac{40}{40-x}$ SUM OF FREE PERIODS OF AVERAGE ENZYME MOLECULE. TIME SPENT IN COMBINING WITH UREA	$\frac{x}{Ed}$ SUM OF PERIODS SPENT BY ENZYME MOL. COMBINED WITH UREA. TIME SPENT IN DECOMPOSING UREA	$\frac{1}{E}\left(\frac{1}{c}\log\frac{40}{40-x}+\frac{x}{d}\right)$ TOTAL TIME CALCULATED FROM x	ACTUAL TIME
	minutes	*minutes*	*minutes*	*minutes*
14.08	10.9	9.2	20.1	20
20.22	17.7	13.2	30.9	30
25.06	24.7	16.3	41.0	40
29.20	32.9	19.0	51 9	51
31.98	40.8	20.8	61.6	60
35.12	52.8	22.9	75.7	75
36 90	64.1	24.1	88.2	90
38.00	75.2	24.8	100.0	105
39.00	92.6	25 3	117.9	120
39.54	112.1	25.8	137.9	135

The relatively rapid increase of the figures in the second column compared with those in the third shows how the proportion of the time consumed by enzyme and urea in combining increases

as the urea concentration becomes smaller with the progress of the reaction.

The effect of urea concentration on combining velocity is seen still more plainly when one considers the figures of Experiment 3, where the urea concentration varies over a much greater range.

TABLE IX.

Analysis of results of Experiment 3.

INITIAL UREA CONCENTRATION		x	$189 \log \frac{a}{a-x}$ SUM OF FREE PERIODS. TIME CONSUMED IN COMBINING	$1.935\,x$. SUM OF COMBINED PERIODS. TIME CONSUMED FOR DISRUPTION	TOTAL TIME, CALCULATED FROM a AND x	ACTUAL TIME
Per cent	a					
0.0375	12.5	5.8	51.2	11.2	62.4	60
0.075	25.0	10.4	44.0	20.1	64.1	60
0.15	50.0	15.5	30.4	30.0	60.0	60
0.30	100.0	21.2	18.5	41.0	59.5	60
0.60	200.0	24.6	10.8	47.6	58.4	60
1.2	400.0	27.0	5 7	52.3	58.0	60
2.4	800.0	28.5	2.9	55.2	58.1	60
4.8	1600.0	31.0	1.6	60.0	60.1	60
9.6	3200.0	31.0	1.0	60.0	61.0	60

The accuracy with which the equation fits all the conditions met in the above experiments is indication that it expresses the real mechanism of the enzyme action.

Following are certain points of interest in connection with the application of the equation and interpretation of results obtained with it.

1. Effect of enzyme concentration. The time required for the decomposition of a given amount of urea varies inversely as the enzyme concentration. Or, the velocity is directly proportional to the enzyme concentration.

2. Form of time curve of the reaction. The reaction in all its possible stages is represented by a curve which starts as a straight line, but gradually conforms to the logarithmic curve $t = \frac{1}{c} \log \frac{a}{a-x}$ as the factor $\frac{a}{a-x}$ becomes larger. Towards the end of the reaction this term predominates, and the curve approximates that of the simple mass action formula, $t = \frac{1}{K} \log \frac{a}{a-x}$. Reactions of

that nature, represented by straight lines passing into logarithmic curves, have already been noted in the case of invertase by Adrian Brown,[7] and of diastase by Horace Brown and T. A. Glendinning.[8] The quantitative application of the equation to the results of these authors will be taken up later in this paper.

3. Determination of d. d is determined from the initial velocity of the reaction, the substrate concentration being high enough to give this velocity its maximum value. In order to determine this concentration, it is necessary to determine the initial velocity with a series of solutions of increasing substrate concentration. When the latter reaches such a height that the combination of enzyme and substrate is practically instantaneous, compared with the rate of disruption of the combination, the term $\frac{1}{c}\log\frac{a}{a-x}$ in the equation $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$ becomes negligible, the reaction rate becomes independent of the urea concentration and the entire equation simplifies to $t = \frac{x}{Ed}$ or $d = \frac{x}{Et}$. Experiment 3 serves as an example of the method for determining the concentration of substrate that must be used to ascertain d.

It should be noted that even when the time curve of the reaction starts as a practically straight line, the initial velocity is not necessarily the maximum, Ed, as the logarithmic curve itself may be fairly straight at the start. Figure 3, for example, shows curves starting as nearly straight lines, while the tabulated analysis of the results (see table VIII,)shows that even at the start about as much of the time consumed is represented by the term $\frac{1}{c}\log\frac{a}{a-x}$ as by the term $\frac{x}{d}$; *i.e.*, the average enzyme molecule here spends as much time free and therefore inactive as it does in combination with urea. The reduction of the inactive period to zero depends on having a sufficient concentration of substrate, so that the enzyme after splitting off one substrate molecule loses only a negligible amount of time in getting into touch with another.

[7] *Trans. Chem. Soc.*, lxxxi, p. 373, 1902.
[8] *Ibid.*, lxxxi, p. 388, 1902.

The relations come out clearly when one considers equation (2),

$$\frac{dx}{dt} = E\frac{c\,du}{d+cu}$$

When u (urea concentration) is increased to such an extent that d is comparatively negligible in comparison with cu, the velocity approximates the value

$$\frac{dx}{dt} = E\frac{c\,du}{cu} = E\,d.$$

The fact that we are able directly to determine d in the case of urease by the use of practicable substrate concentrations must be considered a matter of chance. If urea were less soluble, or the ratio $\frac{c}{d}$ smaller, d could be determined only indirectly by calculation or extrapolation.

d having been determined, c is calculated from independent data by equation (9):

$$c = \frac{d}{d\,t\,E-x}\log\frac{a}{a-x}.$$

4. The effect of reaction products. The equation $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$ expresses only the effect of enzyme and substrate concentrations on the course of the reaction. It contains no factors expressing the influence of the products. It holds, therefore, only when the products do not affect the activity of the enzyme. In the case of urease this condition is attained by using a neutral buffer mixture to prevent the development of an alkaline reaction in the solution. The development of the equation to include the effect of reaction products will be taken up in the next paper.

5. Determining the nature of the effect of inhibiting substances. Inhibiting substances can:

(*a*) Interfere with combination of enzyme and substrate, in which case the nature and degree of the effect is shown by a depression of c (see next paper for examples).

(*b*) Interfere with the hydrolytic action of the enzyme after it has combined with the substrate. In this case d will be depressed.

(*c*) Destroy a portion of the enzyme entirely. Both constants will be depressed in the same proportion.

It is evident that the equation can serve to analyze the effect of inhibiting substances on the enzyme.

6. Significance and relation of the numerical values of c and d in the equation $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x}+\frac{x}{d}\right)$. The numerical values of c and d respectively can not be directly compared. d represents the *amount* of substrate decomposed in unit time when the inactive periods of the enzyme (*i.e.*, the intervals spent uncombined with urea) are negligible. The numerical value of d therefore varies inversely as the size of the units in which x is expressed.

c, on the other hand, represents the *proportion* of substrate present which would combine in unit time with unit enzyme having all its combining groups free, and is therefore independent of the terms in which x is expressed. It is an ideal constant, which, theoretically, can represent the rate of combination only during the first instant of the reaction. After this, all the combining groups are never free, because disintegration of the enzyme-urea combinations is not instantaneous. If it were, or were very rapid in comparison to combination, c would approximately represent the fraction of substrate decomposed per time unit, and the equation would assume the monomolecular form, $c = \frac{1}{t}\log\frac{a}{a-x}$. This condition can be approximated in the case of urease by maintaining a slight acidity ($H^+ = 10^{-5.9}$) in the medium. The rate of combination is so much depressed that disintegration, although slightly depressed itself, becomes relatively very rapid (see Table XIV, next paper).

Relation of our equation to similar ones derived from the action of other enzymes.

Invertase and emulsin. Work of Henri.[9] It is an interesting fact that the formulation of a different conception of enzyme kinetics has led Henri, who was apparently assisted by Bodenstein, to an equation almost identical with the above (7), the

[9] *Zeitschr. f. physikal. Chem.*, xxxix, p. 194, 1902.

reasoning being based on results obtained with invertase. Assuming that the enzyme-substrate compound $[ES]$ is formed instantaneously, but is in continuous equilibrium with free enzyme, represented by $E-[ES]$, and with substrate, $(a-x)$, (for the sake of uniformity we give Henri's reasoning in symbols similar to those used in the preceding section) we should have, according to the law of mass action:

$$\frac{\text{Bound enzyme}}{\text{Free enzyme}} = \frac{[ES]}{E-[ES]} = c\,(a-x)$$

$$[ES] = \frac{c\,E\,(a-x)}{1+c\,(a-x)}$$

Assuming that the velocity of enzyme action is proportional to the amount of enzyme $[ES]$ in combination with substrate, we have

$$\frac{dx}{dt} = E\,\frac{d\,c\,(a-x)}{1+c\,(a-x)}$$

Here d represents, as in our derivation, the velocity of decomposition of the enzyme-substrate complex. c, however, represents in this case the ratio, concentration of enzyme combined with substrate to concentration of that uncombined, assuming that cleavage of the substrate united to an enzyme molecule is instantly followed by combination with fresh substrate, so that equilibrium conditions are maintained. This assumption is not made in our derivation, c representing merely the speed with which combination of enzyme and substrate occurs.

Integration of the above velocity equation gives

$$t = E\left(\frac{1}{c\,d}\log\frac{a}{a-x} + \frac{x}{d}\right)$$

The only difference between this equation and (7) is that here the coefficients of *both* terms on the right side are assumed to be inversely proportional to d, so that any influence affecting the value of d must affect the value of cd to an equal proportion. In the next paper, it will be seen, however, that 30 per cent alcohol depresses the second coefficient, d, 50 per cent, while the first coefficient is depressed only 30 per cent. The explanation could be offered that the alcohol increases c at the same time that it depresses d, but that seems improbable. Consequently we are

inclined to believe that the action follows the mechanism assumed in our derivation: *viz.*, combination, taking its own share of the time, and decomposition, also taking its share. We intend, however, to study more thoroughly the effect of different influences on the coefficients.[10] As the influence of products on this enzyme can be obviated, urease offers particularly clean-cut results bearing on this point.

Henri found that his equation held not only with invertase, but also with emulsin acting on salicin. His experimental work has been shown by Hudson[11] to be subject to criticism because Henri in his polarimetric observations did not take into account the mutarotation of glucose. When the effect of the mutarotation of glucose was obviated by adding sodium carbonate to the solutions before polarizing them, Hudson found that in the cases *both of invertase, and emulsin acting on salicin, the ordinary* formula $t = \frac{1}{K} \log \frac{a}{a-x}$ held accurately. Hudson's results are beyond criticism, but it appears that conditions of acidity under which he worked caused an approximation to the formula $t = \frac{1}{K} \log \frac{a}{a-x}$, which does not hold generally. During the course of reaction in a single solution, if the ratio $\frac{c}{d}$ is small enough, the two equations $t = \frac{1}{K} \log \frac{a}{a-x}$ and $t = \frac{1}{c} \log \frac{a}{a-x} + \frac{x}{d}$ may give very similar curves until nearly all of the substrate has been decomposed. In our Experiments 4, 5 and 6, for example, the equation

[10] Henri's actual differential equation contained an added term in the denominator to express the retarding influence of the product, x, on the velocity, the product being assumed to bind a portion of the enzyme. His differential was consequently $\frac{dx}{dt} = \frac{K(a-x)}{1 + m(a-x) + nx}$, yielding the integrated equation $t = \left((1 + an) \log \frac{a}{a-x} + (m-n)\, x\right)$. As the product in moderately dilute neutral solution does not retard at all the action of urease, this factor does not apply in our case, and we have left it out in order to show more clearly the similarity of the conclusions arrived at by the two somewhat different methods of reasoning.

[11] Hudson: *Journ. Amer. Chem. Soc.*, xxx, pp. 1160 and 1564, 1908; xxxi, p. 1242, 1909.

$K = \frac{1}{t} \log \frac{a}{a-x}$ gives fairly constant results over the early part of the reaction time, although K shows a rapid rise later.

In the next paper it will be shown that the ratio $\frac{c}{d}$ for urease varies greatly with the hydrogen ion concentration, becoming so low in slightly acid solutions that the equation approximates closely to $t = \frac{1}{K} \log \frac{a}{a-x}$. Hudson also worked with acid solutions, and Sörensen[12] has shown that as a matter of fact the monomolecular formula gives constant results with invertin only when $H^+ = 10^{-4}$. With less acid solutions the constant K increases as the reaction progresses; with more acid it falls.

When *different initial concentrations* of substrate over a sufficient range are taken (as in Exp. 3), the value of K in the ordinary equation is found to vary, even when conditions are such that it is constant during the course of reaction in a single solution. Thus, the results of O'Sullivan and Tompson with invertase[13] by the same methods used by Hudson, show fairly constant results for $K = \frac{1}{t} \log \frac{a}{a-x}$ through the course of the reaction with a given initial sugar concentration. When that concentration is varied however, the value of K varies inversely with it, as pointed out by Adrian Brown.[14] The experimental work of Barendrecht cited in the next paragraph is free from the inaccuracy of that of Henri, and yet shows that the action of invertase follows an equation of the general form derived by Henri, the simple formula $t = \frac{1}{K} \log \frac{a}{a-x}$ failing to express the results.

Lactase and invertase. Barendrecht,[15] working with invertase and lactase, assumed that the enzyme divides its energies between substrate and products in proportion to their concentration. In explaining this he introduces the apparently unnecessary idea

[12] S. P. L. Sörensen: Enzymstudien, *Biochem. Zeitschr.*, xxi, pp. 275–79, 1909.

[13] *Trans. Chem. Soc.*, lvii, p. 865, 1890.

[14] *Ibid.*, lxxxi, p. 373, 1902.

[15] Barendrecht: *Zeitschr. f. physik. Chem.*, xlix, p. 456, 1904; *Biochem. Journ.*, vii, p. 559, 1913.

that the enzymes act through radiations which are absorbed by substrate and products. His basic assumption, of the distribution of enzyme energy between substrate and products, is similar to Henri's, however, and leads to an outwardly similar equation,

$$t = \frac{a\,n}{m} \log \frac{a}{a-x} + \frac{1-n}{m}\,x.$$

That the equation with the constants assumed can not hold rigidly, however, is seen from the following considerations. If the effect of the products, represented by the constant n, be zero, as with urease in neutral solution, the equation becomes

$$t = \frac{x}{m},$$

a straight line from start to finish of the reaction. Such would be contrary to the law of mass action and to all observed facts in time reactions. The case of urease is one in point. We have an unretarded reaction, which is not linear.

For a given substrate concentration, however, Barendrecht's equation has the same form as Henri's and ours. Barendrecht's careful experimental work (the accurate reduction method of Kjeldahl was used instead of the polarimetric) shows that with a given initial substrate concentration of either lactose or sucrose the equation does hold throughout the course of the reaction. The work of Barendrecht, therefore adds lactase to the enzymes found to follow the general law $t = m \log \frac{a}{a-x} + n\,x$. Barendrecht's results with invertase also fit Henri's formula, and corresponding to the greater exactness and accuracy of Barendrecht's methods, more accurately than the data of Henri himself.

Maltase. Mlle. Piloche[16] found that the action of maltase (Taka-diastase) on different concentrations of maltase follows the equation of Bodenstein, $t = \frac{1}{K}\left(2\,x + a \log \frac{a}{a-x}\right)$, which is of the same general form as Henri's. With diastase acting on starch and glycogen a definite formula could not be applied. It is possible that some unrecognized side reaction obscured the main enzyme effect, as Brown and Glendinning obtained results with

[16] Piloche: *Journ. de chim. et de phys.*, vi, p. 254, 1908.

malt extract which entirely conform to the general equation $t = m \log \frac{a}{a-x} + n x$.

Diastase. Brown and Glendinning have noted in the action of diastase on starch a time curve similar in form to that representing the action of urease in neutral solutions. As they state it: "We are able to analyze our time curves and divide them into two parts, an earlier one which is *linear*, and a later one which is approximately *logarithmic*."[17]

The following table shows that their results are quantitatively expressed by the equation $t = \frac{1}{c} \log \frac{a}{a-x} + \frac{x}{d}$. As no experiments were performed from which d can be derived independently, we have to calculate it from two different points on the curve, taking that value for d which gives the same c at both points. The value found for d is 0.024. Using this value in the equation $c = \frac{d}{dt-x} \log \frac{a}{a-x}$, we find the following values for c. In this case $a = 1$.

TABLE X.

Action of diastase on soluble starch.

Data of Horace Brown and Glendinning.

t	x	c
10	0.1084	0.0091
20	0.2250	0.0103
30	0.335	0.0110
40	0.435	0.0113
50	0.535	0.0120
60	0.615	0.0119
70	0.680	0.0119
80	0.738	0.0119
90	0.780	0.0115
100	0.815	0.0111
110	0.850	0.0110
120	0.880	0.0110
130	0.903	0.0113
140	0.922	0.0109
150	0.940	0.0110
160	0.950	0.0108

[17] *Trans. Chem. Soc.*, lxxxi, p. 388, 1902.

The constancy of c shows that the entire course of the reaction follows the formula as closely in the case of diastase as in that of urease.

Peptase. Abderhalden and Michaelis[18] have plotted the course of the cleavage of *d*-alanyl-*d*-alanine by yeast juice, from results of Aberhalden and Koelker.[19] Abderhalden and Michaelis found that the course of the reaction is too much retarded, as it progresses, for the straight line equation $\frac{x}{t} = K$, and not enough retarded for the ordinary first order equation $\frac{1}{t} \log \frac{a}{a-x} = K$. They have, therefore, struck an average by adding the two equations together and thus obtained the equation:

$t = \frac{1}{K} \log \frac{a}{a-x} + \frac{\epsilon}{K} x$, which fits the results.

This equation differs from our equation, $t = \frac{1}{E}\left(\frac{1}{c} \log \frac{a}{a-x} + \frac{x}{d}\right)$ only in that it does not express the proportionality between enzyme concentration and velocity, and that like Henri's equation it assumes a proportionality between c and d. The formula, however, being merely the average between two others, neither of which fit the case, the authors attached to it at the time no theoretical or general interest, although they were aware of its relation to Henri's formula.

The results of all four tables of Abderhalden and Michaelis give the same average c calculated by our equation $c = \frac{d}{dtE-x} \log \frac{a}{a-x}$, d being taken as 0.020, E representing the cc. of enzyme solution added.

Effect of varying urease concentration in absence of neutrality regulator.

The preceding experiments have shown that when the alkaline effect of the generated ammonium carbonate is prevented by a neutral phosphate mixture, the reaction rate is directly proportional to enzyme concentration. In the following experi-

[18] *Zeitschr. f. physiol. Chem.*, lii, p. 326, 1907.
[19] *Ibid.*, li, p. 294, 1907.

ment the same proportionality is demonstrated without the presence of neutrality regulator. The time is here varied inversely as the enzyme concentration, so that if the rule, velocity proportional to catalyst concentration, holds, the amount of ammonia formed will be constant, and the effect of its alkalinity also constant.[20]

Experiment 7. Action of enzyme on pure urea. Time inversely proportional to enzyme concentration. Other conditions constant. Urea concentration, 5 per cent; volume, 8 cc.; temperature, 20°.

TABLE XI.

ENZYME CONCENTRATION IN CC. 0.5 PER CENT UREASE	TIME	$\frac{N}{50}$ NH_3 FORMED
	hours	*cc.*
16	$\frac{1}{8}$	18.6
8	$\frac{1}{4}$	18.5
4	$\frac{1}{2}$	17.9
2	1	18.3
1	2	17.9

The rate of urea decomposition is exactly proportional to enzyme concentration. Our previous experiments have shown that this law holds when the alkaline effect of ammonium carbonate is prevented by neutral phosphate mixture. This experiment shows that the rule holds also when the ammonium carbonate factor is not eliminated, but kept constant.

The following experiment shows that the rule does not hold closely when the ammonium carbonate formed is allowed to vary, the time being kept constant with variable enzyme.

[20] This experiment formed part of the work reported at the December meeting of the American Society of Biological Chemists. Marshall had at that time already performed practically identical experiments which have since been published in the April number of this *Journal*. Our results correspond entirely with Marshall's.

Experiment 8. One cc. of 25 per cent urea was added to 4 cc. of enzyme solution of varying concentration. Time constant at fifteen minutes; temperature 20°.

TABLE XII.

RELATIVE ENZYME CONCENTRATION E	$\frac{N}{50}$ NH_3 FORMED IN 15 MINUTES	$\frac{NH_3}{E}$
	cc.	
64	192.0	3.00
32	107.0	3.35
16	58.0	3.62
8	34.0	4.25
4	19.5	4.88
2	10.1	5.05
1	5.2	5.20

With the lower enzyme concentrations the reaction rate is approximately proportional to the enzyme concentration. The larger amounts of enzyme, however, decompose considerably less than the corresponding amounts of urea (see last column). The larger amounts of ammonium carbonate formed in the first few minutes in these cases cause a greater depression of the enzyme's activity. The quantitative nature of this depressing effect of the alkaline carbonate is taken up in the next section.

Empirical formula expressing influence of generated ammonium carbonate on reaction velocity.

As Armstrong and Horton have shown, the ammonium carbonate formed from urea retards the enzyme action, so that the curve of ammonia formation progressively decreases its slant until the latter finally becomes constant. While the ammonium carbonate retards the reaction, additional amounts have less and less effect, until finally the reaction approaches a constant rate, practically unaffected by further carbonate formation so long as a sufficient excess of urea remains.

In the following paper the relation of the depressing effect of the carbonate to its alkalinity is demonstrated. Here, however, we merely develope an empirical formula which has proven useful in studying the action of the enzyme in solutions devoid of neutralizing substances.

In attempting to express the relations mathematically we proceeded on the assumption that the enzyme was changed by the ammonium carbonate from its original form E_m in which it reacted with the velocity m, to a final form E_n in which it reacted with the velocity n (n was found equal to $\frac{m}{7.7}$), the ratio of E_n to E_m being proportional to the ammonium carbonate concentration. Representing the NH_4 concentration as x, these relations are expressed by the equation:

$$\frac{E_m}{E_n} = \frac{K}{x} \tag{1}$$

Representing the total enzyme concentration as E, we have:

$$E_n + E_m = E \tag{2}$$

The velocity of the reaction is expressed by the differential:

$$\frac{dx}{dt} = m(E_m) + n(E_n) \tag{3}$$

(t = time of reaction).

Combining the above three equations to eliminate E_m and E_n, we have

$$\frac{dx}{dt} = \frac{E(Km + nx)}{K + x} \tag{4}$$

Integrating, and finding the constant of integration by placing t and x equal to 0, we have:

$$t = \frac{1}{E}\left(\frac{x}{n} - \frac{K(m-n)}{0.4343\,n^2}\log_{10}\frac{Km + nx}{Km}\right) \tag{5}$$

The equation is simplified if we chose as unit enzyme concentration ($E = 1$) that which corresponds to unit value for n; *i.e.*, that enzyme concentration which will make the final rate of ammonia formation 1 cc. of $\frac{N}{1}$ NH_3 per minute per liter of solution. We then have:

$$t = \frac{1}{E}\left(x - \frac{K(m-1)}{0.4343}\log_{10}\frac{Km + x}{Km}\right) \tag{6}$$

With this equation results can be calculated about as rapidly as the ordinary analytical proportions, and they hold with con-

siderable accuracy for the enzyme concentrations ordinarily used. The ratio between m and n was found by extrapolation of the observed velocities to be 7.7:1; the value of K was found by substitution to be 30. Substituting 1 for n, 7.7 for m and 30 for K, we have for temperature 20°,

$$t = \frac{1}{E}\left(x - 462 \log_{10} \frac{231 + x}{231}\right) \tag{7}$$

or

$$E = \frac{1}{t}\left(x - 462 \log_{10} \frac{231 + x}{231}\right) \tag{8}$$

The following series of experiments on the reaction rate indicate the degree of accuracy with which the equation holds under varying conditions of enzyme concentration and reaction time.

The soy bean extract used as enzyme solution was in each case standardized as follows: 1 cc. of extract at 20° was mixed with 5 cc. of 5 per cent urea at the same temperature; after exactly fifteen minutes the reaction was checked, in the manner previously described, by adding potassium carbonate solution without opening the tube. The ammonia was immediately aerated as usual. From the values of x thus found (x = cc. $\frac{N}{1}$ NH_3 formed per liter of reacting solution $= \frac{20}{6}$ the cc. of $\frac{N}{50}$ ammonia titrated) the enzyme concentration per cc. of reacting solution is calculated: $E = \frac{1}{15}\left(x - 462 \log \frac{231 + x}{231}\right)$. As the extract is diluted with 5 volumes of urea solution the enzyme concentration in the extract itself is six times the value thus found. For brevity the standardization data will be given in the following form:

Extract activity (− cc. $\frac{N}{50}$ NH_3), E = −.

The volume of $\frac{N}{50}$ HCl neutralized is given in parentheses, and E represents the enzyme concentration in the undiluted extract calculated as above described. An enzyme solution of concentration E = 1, in the above units, corresponds approximately to a 1.6 per cent solution of solid urease.

Experiment 9. Very dilute enzyme, $E=0.048$. Ten cc. of extract added to 1000 cc. of 1 per cent urea. Samples of 100 cc. at intervals for ammonia determination by vacuum distillation. Temperature, 20°. Extract activity (16.62 cc. $\frac{N}{50}$ NH_3), $E=4.92$. For 101-fold dilution, $E=\frac{4.92}{101}=0.048$.

TABLE XIII.

t	$\frac{N}{50}$ HCl NEUTRALIZED BY 100 CC. SOLUTION	x ($\frac{N}{1}$ NH_3 PER LITER)	E CALCULATED FROM x	t CALCULATED ON BASIS $E=0.048$
minutes	*cc.*			
10	17.1	3.43	0.052	10
15	23.1	4.62	0.043	13
60	75.8	15.16	0.040	50
120	141.0	28.20	0.042	106
180	196.0	39.20	0.044	165
240	243.0	48.60	0.043	214

Experiment 10. $E=0.257$. Enzyme extract diluted three times. One cc. portions of diluted enzyme added to 5 cc. portions of 5 per cent urea. Usual technique. Temperature, 20°.

Extract activity (15.8 cc. $\frac{N}{50}$ NH_3); $E = 4.62$

For 18-fold dilution, $E = \frac{4.62}{18} = 0.257$

TABLE XIV.

t	$\frac{N}{50}$ HCl NEUTRALIZED BY 6 CC. SOLUTION	x ($\frac{N}{1}$ NH_3 PER LITER)	E CALCULATED FROM x	t CALCULATED ON BASIS $E=0.257$
minutes	*cc.*			
15	6.21	20.7	0.230	13
30	10.46	34.9	0.220	30
60	18.20	60.6	0.230	53
120	30.50	101.6	0.236	110
240	51.90	173.2	0.252	235
480	76.60	278.0	0.220	446
960	140.00	464 0	0.254	950

Experiment 11. $E = 1.07$. Conditions same as in preceding experiment, except that 1 cc. of *undiluted* extract was used for each portion. The extract used was also somewhat stronger in this case.

Extract activity (20.0 cc. $\frac{N}{50}$ NH_3); $E = 6.42$

Extract 6-fold diluted, $E = \frac{6.42}{6} = 1.07$.

TABLE XV.

t	$\frac{N}{50}$ HCl NEUTRALIZED	x ($\frac{N}{1}$ NH_3 PER LITER)	E CALCULATED FROM x	t CALCULATED ON BASIS $E = 1.07$
minutes	*cc.*			
5	9.06	30.2	1.10	5.2
10	14.7	49.0	1.03	9.7
15	20.0	67.4	1.07	15.0
30	34.0	112.0	1.09	30.5
45	43.9	146.2	1.06	44.7
60	53.4	177.8	1.05	59.0
120	89.6	298.6	1.10	124.0
180	121.0	400.0	1.10	186.0
240	145.0	483.0	1.07	140.0
300	168.0	560.0	1.04	294.0
360	192.0	644.0	1.05	354.0
		Average = 1.069		

Experiment 12. $E = 5.70$. Highest enzyme concentration. Four cc. of 1 to 5 extract added to 1 cc. of 25 per cent urea. Temperature 20°.

Extract activity (18.7 cc. $\frac{N}{50}$ NH_3); $E = 5.70$

Extract $\frac{4}{5}$ diluted, $E = 4.56$.

TABLE XVI.

t	0.102 N HCl NEUTRALIZED	x ($\frac{N}{1}$ NH_3 PER LITER)	E CALCULATED FROM x	t CALCULATED ON BASIS $E = 4.56$
minutes	*cc.*			
5	4.70	94	5.11	5.5
10	8.35	167	5.85	12.7
15	11.25	225	5.92	19.4
30	17.10	342	5.49	35.0
60	27.40	548	5.12	67.0
90	37.40	748	5.13	100.0
120	44.10	883	4.75	124.0
180	57.50	1151	4.43	174.0
240	69.70	1394	4.21	220.0
		Average = 5.11		

Experiment 13. $E = 0.36$ at 20°. Reaction at 40°. One to 5 extract diluted three times. One cc. of diluted enzyme added to 5 cc. of 5 per cent urea.

TABLE XVII.

t	$\frac{N}{50}$ HCl NEUTRALIZED	x ($\frac{N}{1}$ NH_3 PER LITER)	E (40°) CALCULATED FROM x	t CALCULATED ON BASIS $E = 1.30$
minutes	cc.			
5	11.83	38.4	1.58	6.1
10	16.33	54.0	1.20	9.2
15	23.38	76 8	1.21	14.0
30	39.33	131.0	1.36	32
60	65.25	217.6	1.40	65
180	132.20	440.8	1.30	180
240	166.60	555.0	1.29	238
300	178.50	598.0	1.14	263

The average value of $E_{40°}$ is approximately that calculated from $E_{20°}$ (activity at 20°) by the temperature coefficient ascertained in Experiment 14. The coefficient given:

$$\log \frac{E_{40°}}{E_{20°}} = 0.029\ (40 - 20) = 0.58$$

$$E_{40°} = 3.8\ E_{20°} = 3.8 \times 0.36 = 1.37$$

The above results indicate the degree of accuracy of the formula over a wide range of enzyme concentration and time interval. The results of Armstrong and Horton[21] fit the formula with about equal accuracy.

TABLE XVIII.

Results of Armstrong and Horton recalculated, temperature=25°.

t	x	$E_{25°}$	t CALCULATED ON BASIS $E = 1.75$
minutes			
5	42	1.66	4.7
15	94	1.64	14
30	156	1.75	30
45	208	1.75	45
60	257	1.78	63
180	618	1.98	204
300	854	1.81	310
420	1084	1.74	419

[21] *Proc. Royal Soc.*, lxxxv, p. 116, 1912.

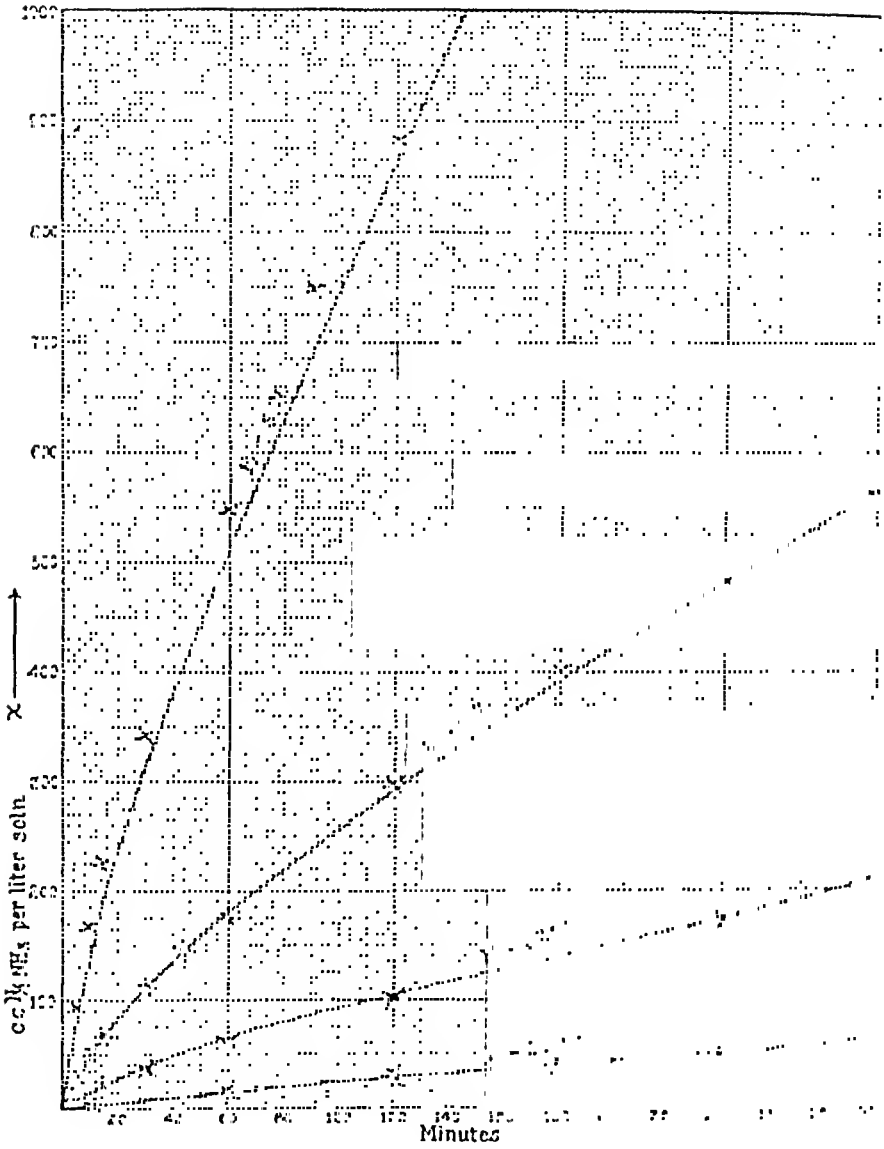


FIG. 4. From experiments 9, 10, 11 and 12. Action of different concentrations of urease in absence of neutrality regulator. Curves are plotted from values of t calculated by empirical equation, $t = \frac{1}{E}\left(x - 462 \log \frac{231+x}{231}\right)$. Crosses represent experimental results.

It is evident that, while this empirical formula has not the theoretical interest of the one developed on the basis of the law of mass action, yet it fits a very wide range of enzyme concentrations with sufficient accuracy to make it useful where the mass action formula can not be applied, viz., when there is no neutrality regulator present to keep the H^+ constant. This formula, unlike

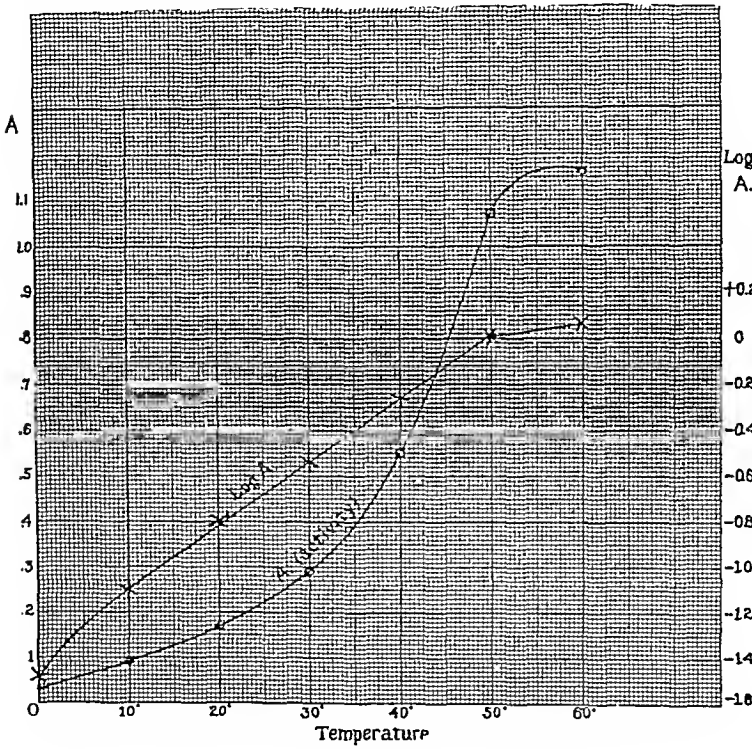


FIG. 5. From experiment 14. Effect of temperature on enzyme activity, *A*.

the theoretical one, does not hold for the last stage of the reaction, but applies only when the urea concentration remains high enough (above about 0.08 per cent) so that variations in it do not affect the enzyme. This concentration is, however, so low that the equation holds nearly to the finish of the reaction (see Exp. 2),

and about 95 per cent of the urea present is decomposed in the time calculated by the equation for 100 per cent (see Fig. 1 for example).

The formula fits most accurately when the enzyme concentration is in the neighborhood $E = 1$, as the constants of the equation were determined with this concentration. It is about that of a 1.6 per cent solution of the urease described in the second following paper.

Effect of temperature on rate of the urease action.

Temperature coefficient. Experiment 14. Five cc. portions of 5 per cent urea solution were brought to the desired temperatures in water baths, 1 cc. of urease solution added to each, and the reaction stopped with potassium carbonate in the usual manner after fifteen minutes; the ammonia was determined by aeration. For the temperatures up to 20° a 1:5 extract of soy bean meal was used. For temperatures above 20° the same extract was diluted five-fold, in order to avoid formation of excessive amounts of ammonia The activity a is calculated from formula (S) above, A being substituted for E in the isotherm, $E = \frac{1}{15}\left(x - 462 \log \frac{231 + x}{231}\right)$.

TABLE XIX.

Temperature coefficient.

ENZYME SOLUTION USED	TEMPERATURE	$\frac{N}{50}$ HCl NEUTRALIZED	x ($\frac{N}{1}$ NH_3 PER LITER)	ACTIVITY A	TEMPERATURE COEFFICIENT FOR 10° INTERVAL
	deg.	*cc*			
1 to 5 extract	0	4.54	15.2	0.16	
					>2.80
1 to 5 extract	10	10.71	35.7	0.45	
					>2.00
1 to 5 extract	20	17.90	59.7	0.90	
Same extract five times diluted	20	4.24	14.1	0.16	
					>1.81
Same extract five times diluted	30	7.42	24.7	0.29	
					>1.90
Same extract five times diluted	40	12.20	40.7	0.55	
					>1.95
Same extract five times diluted	50	20.30	67.7	1 07	
					>1.00
Same extract five times diluted	60	21.30	71.0	1.16	

The average temperature coefficient is 1.91, about that usually found in chemical reactions. As it is nearly constant between 10 and 50°, the usual logarithmic temperature formula can be applied. Representing the activity at any temperature t from 10° to 50° by A_t, we have:

$$\log A_{t_1} = \log A_{t_2} + 0.029\ (t_1 - t_2).$$

Heat stability of the urease. Experiment 15. A five-fold diluted extract was heated, then 1 cc. allowed to act for fifteen minutes on 5 cc. of 5 per cent urea at the same temperature. In a control determination the enzyme was brought to this temperature as quickly as possible, and then at once added to the urea.

TABLE XX.

TEMPERATURE	PERIOD OF PRELIMINARY HEATING	$\frac{N}{50}$ HCl NEUTRALIZED BY NH_3 FORMED	$\frac{N}{50}$ HCl NEUTRALIZED IN CONTROL WITHOUT PRELIMINARY HEATING
deg.	*minutes*	*cc.*	*cc.*
60	30	24.36	24.15
70	30	15.74	20.56
80	30	0.85	18.8

From these figures it is evident that the enzyme in water solution is not affected by 30 minutes exposure at 60°, loses about one-fourth its activity at 70°, and is almost completely destroyed at 80°.

SUMMARY.

1. The action of urease expressed by a mass action formula which is general for enzymes.

Our results indicate that the enzyme destroys urea by means of two successive reactions: (1) Combination of enzyme and urea; (2) Disruption of the combination, the urea being freed as ammonia and carbon dioxide. This process formulated in accordance with the law of mass action leads to the following equation, which is found to hold very accurately.

$$\underbrace{t}_{\text{Time for decomposition of } x \text{ amount of substrate.}} = \underbrace{\frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x}\right.}_{\text{Portion of time consumed in uniting enzyme and substrate. Summation of intervals spent by average enzyme molecule uncombined.}} + \underbrace{\left.\frac{x}{d}\right)}_{\text{Portion of time consumed by enzyme in decomposing substrate. Summation of intervals spent by average enzyme molecule combined with substrate.}}$$

a represents the amount of substrate (urea) present per unit volume at the beginning of the reaction, x the amount decomposed at time t, E the enzyme concentration, c the velocity of combination of enzyme and substrate, and d the velocity at which the combination splits, yielding ammonium carbonate and free enzyme again.

The above application of the law of mass action to enzyme action does not involve the introduction of any arbitrary constant. d as well as c is, in the case of urease at least, capable of experimental determination. Considering this, the constancy with which the equation holds, for urea concentrations varying in ratio from 1 to 300, for enzyme concentrations from 1 to 10, for reaction periods from a few minutes to seven hours, forms strong proof that it expresses the actual mechanism of the enzyme's action.

The usual mass action formula, $t = \frac{1}{K} \log \frac{a}{a-x}$, expresses only time consumed by the first of the two phases of the process and therefore fits only in special cases of enzyme action when the time consumed by the second phase is relatively negligible. The idea that enzymic decomposition consists of two successive time reactions in the case of invertase, and that this is the reason for the failure of the ordinary mass action formula was clearly expressed by Adrian Brown.[22] Up to the present, however, no one appears to have attempted to formulate the course of the dual reaction so that this assumption concerning its nature could be submitted to the test of quantitative application to experimental results.

The formula $K = \frac{1}{t} \log \frac{a}{a-x}$ can be made to hold in special cases by so regulating conditions that the term $\frac{1}{c} \log \frac{a}{a-x}$ in the formula $t = \frac{1}{c} \log \frac{a}{a-x} + \frac{x}{d}$ is very large in comparison to $\frac{x}{d}$. This result can be attained by: (1) Using only such dilute solutions of substrate that the factor $\log \frac{a}{a-x}$ is large as soon as x becomes measurable. (2) By working under such conditions that the

[22] *Trans. Chem. Soc.*, lxxxi, p. 384, 1902.

ratio $\frac{c}{d}$ is relatively small. As will be shown in the next paper, the H^+ concentration has a great influence on this ratio.

Henri, the first to succeed in finding a general application of the law of mass action to enzymes, arrived by a somewhat different mode of reasoning at almost the identical equation obtained by us. Considering the enzyme to be in constant equilibrium with both substrate and products, and the process of the combination itself being instantaneous, he derived the equation:

$$t = \frac{1}{K}\left[(1 + an)\log\frac{a}{a-x} + (m-n)\,x\right]$$

m and n representing respectively the affinities of the enzyme for substrate and products. Henri successfully applied this equation to invertase and emulsin. Although his results as shown by Hudson's accurate investigations are open to criticism because he used the polariscopic method without taking account of the mutarotation of glucose, succeeding work confirms the general applicability of the law of enzyme action which he derived.

In the case of urease we have succeeded in obviating any hindrance of the products (ammonium carbonate) on the action of the enzyme by the use of neutral phosphate. When the products do not retard the action, n in Henri's equation $= 0$, and the equation becomes

$$t = \frac{1}{K}\log\frac{a}{a-x} + \frac{m}{K}x.$$

This differs from our equation only in failing to express the influence of enzyme concentration and in having a common factor, $\frac{1}{K}$, in the coefficients of both terms on the right side. In our equation the two coefficients are represented as quite independent, which seems to be the case.

The elimination of the products as a disturbing factor enables us to avoid the arbitrary cut-and-try method of estimating the constants to which Henri was forced, and we can determine both d and c from independent data. This greatly increases the rigidity of the proof that the formula is theoretically correct rather than an empirical approximation. It also makes urease an enzyme peculiarly fitted for the analysis of enzyme action in general. This is further exemplified in the next paper.

Empirical equations of the same general form as those theoretically derived by Henri and ourselves have been found to hold good by Barendrecht for invertase and lactase, and by Abderhalden and Michaelis for the peptolytic enzyme of the duodenal juice acting on *d*-alanyl-*d*-alanine.

The fact that invertase, maltase, lactase, diastase, emulsin, duodenal peptolytic enzymes, and urease all act in accordance with an equation derived from the law of mass action, indicates that enzymes are not exceptions to that law. Some apparent exceptions may fairly be attributed to conditions which hinder or obscure the action of the enzyme. This is the case with urease, whose product is alkaline, and greatly reduces the activity of the enzyme. When the alkalinity is prevented by a phosphate mixture, urease is seen to follow accurately the general mass action formula of enzymes.

2. Effect of urea concentration on the enzyme action. Definite combining proportions between urea and enzyme.

So long as the concentration of urea remains between 0.08 per cent and 10 per cent, variations in it are, in the absence of other substances, without effect on the rate of ammonia formation.

The sharply defined maximum amount of urea which a given amount of enzyme can hydrolyze per time unit indicates the existence of definite combining proportions between enzyme and substrate. There is no suggestion of the indefinite ratios found in "adsorption" or "absorption."

The reason why so slight a concentration of urea is necessary in order that the enzyme may act with maximum velocity, is that combination between enzyme and urea is so quick that, even in dilute solutions, the enzyme after decomposing one urea molecule losses no appreciable time in combining with the next. In terms of the formula above developed, the ratio $\frac{c}{d} = \frac{\text{combining velocity}}{\text{decomposing velocity}}$ is so great that as long as a certain concentration of excess urea (about 0.08 per cent in the absence of salts, or 4 per cent in the presence of $\frac{M}{2}$ phosphate, which depresses *c*) is present, the time consumed for combination between enzyme

and substrate is relatively negligible, the term $\frac{1}{c}\log\frac{a}{a-x}$ in the equation being too small to add appreciably to the total. Increase of urea concentration beyond this point merely reduces an already negligible factor of time consumption, and consequently does not appreciably accelerate the reaction. *The reaction velocity is, therefore, independent of the urea concentration,* so long as the latter does not sink below the limits mentioned. After it does, the term $\frac{1}{c}\log\frac{a}{a-x}$ plays an increasingly larger rôle in shaping the reaction curve. The result is that the curve, which starts as a straight line if enough urea is present, turns into a logarithmic curve as the urea concentration is reduced towards zero.

When the urea concentration is increased much above 10 per cent, it depresses the enzyme action. High osmotic pressure (that of more than 2 M solutions), whether due to some otherwise inert substance or to the substrate itself, apparently reduces the enzyme's decomposing activity, represented by d in the equation $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$.

3. Course of enzyme action when the alkalinity of the ammonium carbonate formed is not neutralized.

Ammonium carbonate, as above stated, greatly retards the action of urease. The first portions of carbonate formed have the greatest effect, each succeeding portion having less, until after the carbonate concentration has reached about 0.2 molecular the formation of more has hardly an appreciable influence. (The parallelism between the effects of the carbonate on H^+ concentration and enzyme activity respectively, will be taken up in the succeeding paper.) From this point onwards the curve has the form above described, a straight slant ending in a convex curve. The time curve of the complete reaction is therefore convex at the start (because of ammonia retardation), a straight slant in the middle, and convex again at the end (because retarded by decreasing urea concentration). For example, see the lower curve of Fig. 1. When the initial urea concentration is below 0.2 molecular, the first phase runs directly into the last, so that

the curve is convex throughout. The first two phases are expressed by the empirical formula

$$t = \frac{1}{E}\left(x - K \log \frac{c + x}{c}\right)$$

In the absence of salts or other substances particularly influencing the latter part of the reaction, the last phase is so short that the empirical formula holds until the reaction is about 90 per cent complete.

The above formula is a general expression of a reaction gradually changed by the product, x, from a given initial velocity to a given final velocity greater than zero.

4. *Effect of enzyme concentration on reaction rate.*

As has been found independently by Marshall, the velocity of the reaction is directly proportional to the enzyme concentration. We find this true both when the ammonium carbonate is neutralized and when it is not, and the fact is therefore expressed in both the mass action equation and the empirical formula.

5. *Heat stability of the enzyme.*

The enzyme in water solution is not affected by 30 minutes heating at 60°, loses about one-fourth of its activity after 30 minutes at 70°, and is almost completely inactivated at 80°.

6. *Effect of temperature on reaction rate.*

Reaction velocity is nearly doubled by every 10° rise in temperature between 10° and 50°. Within this range the temperature coefficient is nearly constant, and averages 1.91. From 0° to 10° it is 2.80, from 50° to 60° it is only 1.09. The optimum is at about 55°.

The fact that the temperature coefficient is that of a chemical reaction speaks, like the definite amount of urea a given amount of enzyme can hydrolyze per minute, against theories that enzyme and substrate unite by physical means such as adsorption, absorption, diffusion of substrate into colloid enzyme particles, etc. The evidence indicates that enzymes follow the definite laws of chemical reactions.

THE EFFECT OF HYDROGEN ION CONCENTRATION AND OF INHIBITING SUBSTANCES ON UREASE.

FURTHER STUDY ON THE MODE OF ENZYME ACTION.

By DONALD D. VAN SLYKE and GOTTHARD ZACHARIAS.

(*From the Hospital of the Rockefeller Institute for Medical Research, New York.*)

(Received for publication, July 14, 1914.)

It has long been known that some enzymes act best only in alkaline media, others in acid, and still others in neutral, but in recent years the exact work of Sörensen, Michaelis,[1] and Rona has shown that enzymes are in fact so sensitive to the reaction of the solutions in which they are dissolved that an exact study of their modes of action is hardly possible without taking this factor into account and controlling it by accurate methods. With urease this factor is especially important, because the ammonium carbonate which constitutes the reaction product is decidedly alkaline. Consequently in the case of this enzyme the study of the effect of the reaction products is necessarily bound up with that of the effect of hydrogen and hydroxyl ion concentrations.

Armstrong and Horton[2] showed that of the two reaction products, ammonia and carbon dioxide, ammonia retarded the action of the enzyme, while carbon dioxide greatly accelerated it. From a study of the effects of amino-acids and other substances, they came to the conclusion that weak acids as a rule accelerate the action of the enzyme.

Marshall has systematically tested the effect of varying concentrations of hydrochloric acid and sodium hydrate on the action of urease.[3] Hydrochloric acid up to 0.001 N concentration did not depress the amount of ammonia formed in an hour by a

[1] In Oppenheimer: *Handbuch d. Biochemie,* Ergänzungsband, 1913.

[2] Armstrong and Horton: *Proc. Royal Soc.,* lxxxv, p. 118, 1912.

[3] Marshall: this *Journal,* xiv, p. 351, April 1914.

given amount of enzyme acting on an excess of urea, but greater concentrations retarded the action, 0.005 N entirely preventing it. Sodium hydrate in 0.002 N concentration depressed the reaction rate, and the depression increased with alkali concentrations, 0.02 N entirely inhibiting. That the enzyme is destroyed by the higher concentrations of acid and alkali tested was shown by the fact that after neutralization the activity of the enzyme was not restored. The above interesting results do not, however, indicate the H^+ concentration at which the enzyme acts best, nor the quantitative effect of different degrees of deviation from the optimum. The extract contains salts and is rich in proteins, and, as Marshall points out, some of these acting as buffers could neutralize to a large extent the minute amounts of acid and alkali present. The amounts of hydrochloric acid were, furthermore, so small in proportion to the ammonia formed, that the first trace of the latter would neutralize all of the mineral acid, and the reaction would then go on as though none had been added. The initial rate of the reaction would, therefore, in the acid solutions have to be very greatly depressed in order to markedly affect the final result.

In our work we have used phosphate mixtures to fix the hydrogen ion concentrations, and have controlled the latter in every experiment, not only at the beginning, but during the course and at the end of the reaction, by the Sörensen colorimetric method.[4] Only when working in this manner can one accurately interpret results when dealing with a reaction that yields an alkaline product.

The primary purpose of our work was to determine the nature of the effect of the reaction product, ammonium carbonate, on the enzyme. Armstrong and Horton[5] plotted the time curve of the reaction, and showed that the latter runs at a progressively slower rate for some time, after which the rate becomes nearly constant. Ammonium carbonate formation soon depresses the rate to a certain fraction of the initial velocity, but can not depress it much below this fraction. This fact is shown more fully in the preceding paper.

[4] *Ergeb. d. Physiol.*, xii, 1912.

[5] *Proc. Roy. Soc.*, lxxxv, p. 109, 1912.

For this behavior two explanations were possible:

1. The ammonium carbonate has a specific action on the enzyme, which it combines with or changes into a modified form only a fraction as reactive as the unaffected enzyme.

When the ammonium carbonate concentration has increased sufficiently to make the change complete, further formation of ammonium carbonate would naturally have no further effect and the velocity would approach a constant rate, as was found to be the case. Work which is reported in the following pages leaves no doubt, however, that the following explanation is the true one.

2. The ammonium carbonate effect is chiefly one of alkalinity. The carbonate renders the solution too alkaline for optimum activity of the enzyme. The fact that as the ammonium carbonate concentration becomes greater the effect of a given added amount becomes less and less, until finally the reaction rate becomes almost independent of further ammonium carbonate increase, can be explained from the nature of the carbonate as a "buffer" salt. As is well known, the H^+ concentration of solutions of these salts or mixtures (phosphates, acetates, etc.), which owe their acidity or alkalinity to the weakness of either the basic or acid radicle, is but little affected by increasing the concentration beyond a certain point. Both the chemical nature of ammonium carbonate and its effect on the urease lead us to expect from it a similar behavior.

In order to test the point experiments were necessary to determine:

1. The H^+ concentrations of ammonium carbonate solutions in concentrations ranging over those obtained by the enzyme action.

2. The effect on the enzyme of the H^+ concentrations obtained.

The most direct and satisfactory means of testing the first point were offered by determining directly the H^+ concentration of reacting urea-enzyme solutions. Determinations on solutions of so-called "pure" ammonium carbonate are not satisfactory, because the prepared carbonate is a mixture of carbonate, bicarbonate, and carbamate.

H^+ concentrations obtained in reacting solutions of pure urea and enzyme.

One cc. of 10 per cent solution of precipitated urease (method of preparing urease is given in next paper) was added to 100 cc. of 5 per cent urea at 20°. At intervals 5 cc. portions were withdrawn and used for determination of the H^+ concentration by the colorimetric method of Sörensen.

TABLE I.

Change of H^+ concentration as result of formation of ammonium carbonate from urea and urease.

TIME AFTER ADDITION OF ENZYME	P_H	$H^+.10^7$	$OH'.10^7$
Before addition	8.0		
Immediately after	6.5	3.2	0.22
1.5 minutes after	7.6	0.25	2.9
2.5 minutes after	7.9	0.10	7.2
3.7 minutes after	8.1	0.08	9.0
7.0 minutes after	8.5	0 032	22.0
10.0 minutes after	8.7	0.021	34.0
12.8 minutes after	8.8	0.016	45.0
15.5 minutes after	8.9	0.013	55.0
20.0 minutes after	9.0	0.010	72.0
34.0 minutes after	9.08	0.008	89.0
24 hours after	9.34	0.0044	161.0

It will be observed that the enzyme itself has a slightly acid reaction, shown by the increase in the H^+ concentration (decrease in P_H) of the urea solution immediately after its addition. The slight amount of acid substance added with the enzyme is almost immediately neutralized by the ammonium carbonate formed, and the solution becomes progressively more alkaline, but at a decreasing rate.

Simultaneous change of velocity with H^+ concentration during reaction.

In order to determine the velocity changes during the reaction under the conditions of the above experiment, portions of 20 cc.

of 5 per cent urea were mixed at 20° with 1 cc. each of 2 per cent enzyme (concentrations identical with those of preceding experiment). At the end of definite intervals the reaction was checked with carbonate and the ammonia immediately aerated. The technique is detailed in the following paper on urea determination.

TABLE II.

TIME	Δt	$\frac{N}{10}$ NH_3	x* ($\frac{N}{1}$ NH_3 PER LITER)	Δx	VELOCITY $\frac{\Delta x}{\Delta t}$
3′	3′	1 30	1.36	1.36	0.454
6′	3′	2.30	2.41	1.05	0.350
12′	6′	3.65	3.83	1.42	0.236
24′	12′	5.75	6.04	2.21	0.184
80′	56′	15.30	16.06	5.20	0.179

*Calculated for 20 cc. dilution.

These results, with those from the preceding table, are plotted in Fig. 1. They show that increase in alkalinity (increase in P_H) runs parallel with decrease in the reaction rate.

Effect of ammonium carbonate on reaction rate and H^+ concentration in the presence of acid phosphate. Highest rate as solution passes through neutral zone.

By adding primary and secondary phosphate in desired proportions we could both fix the initial H^+ concentration of the enzyme-urea solution, and retard the change in H^+ concentration, so that its relation to the velocity could be followed somewhat more closely than during the extremely rapid changes that occur in absence of phosphate. The enzyme was made to begin its action in an acid medium, and the velocity change observed as the solution gradually passed through neutrality to alkalinity.

Simultaneous determinations of H^+ concentration and reaction rate were carried out in the following solution at 20°.

Solution: 90 cc. $\frac{M}{15}$ KH_2PO_4
10 cc. $\frac{M}{15}$ Na_2HPO_4
3 grams urea
1 cc. 10 per cent precipitated urease

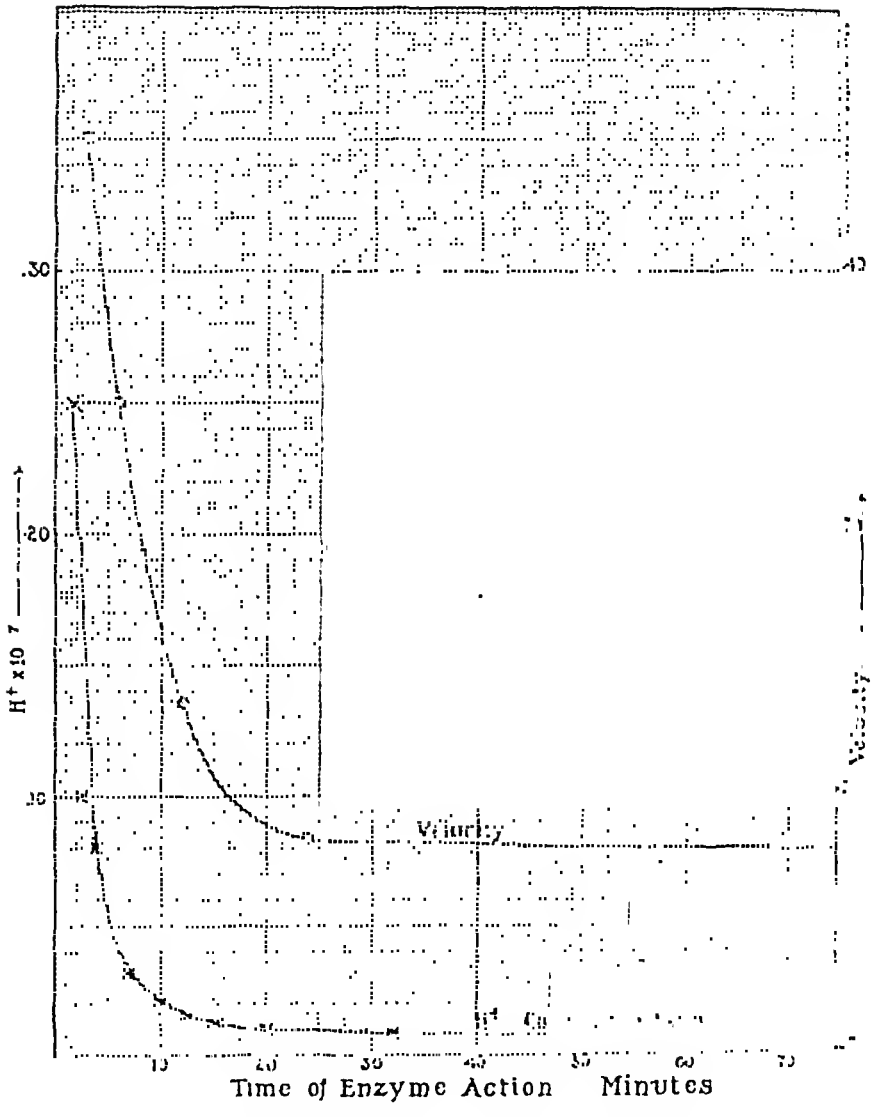


FIG. 1. Fall in velocity with drop in H^+ concentration resulting from formation of alkaline ammonium carbonate. No phosphate present.

For velocity determinations, 10 cc. portions of the mixture were measured out into aerating tubes immediately after addition of the enzyme. The tubes were stoppered and kept at 20° for varying time intervals, at the end of which the respective tubes were treated with carbonate and aerated.

Similar portions, with addition of the proper indicators, served for H^+ determination.

TABLE III.

VELOCITIES						H^+ CONCENTRATION	
TIME IN MINUTES	Δt	$\frac{N}{20}$ NH_3 from 10 cc.	x ($\frac{N}{1}$ NH_3 PER LITER)	Δx	Velocity $\frac{\Delta x}{\Delta t}$	Time in minutes	P_H
		cc.					
44	44	9.45	18.80	18.80	0.427	6	6.07
74	30	18.00	36.00	17 20	0.573	23	6.36
144	70	36.45	72.90	36.90	0.527	45	6.56
204	60	47.05	94.10	21.20	0.353	55	6.64
306	102	61.80	123.60	29.50	0 289	100	6.81
461	155	78.10	156.20	32.60	0.210	135	6.97
						155	7.16
						190	7.35
						230	7.65
						260	7.87
						295	8.17
						305	8.28
						360	8.48
						465	8.75

The influence of the H^+ changes on the velocity is shown by figure 2, in which the curves represent the above data. It will be seen that the velocity rises to its maximum as P_H, representing the alkalinity, increases. The maximum velocity occurs at the time when the P_H curve crosses near the value 7, which is the neutral point at 20°. As alkalinity increases beyond this point, the velocity falls. These data, like those of the preceding experiment, show the effect of an alkaline reaction in depressing the rate of enzyme action. In addition, they show that even a slightly acid reaction also has a depressing effect, the highest activity being observed as the solution passes through the neutral zone.

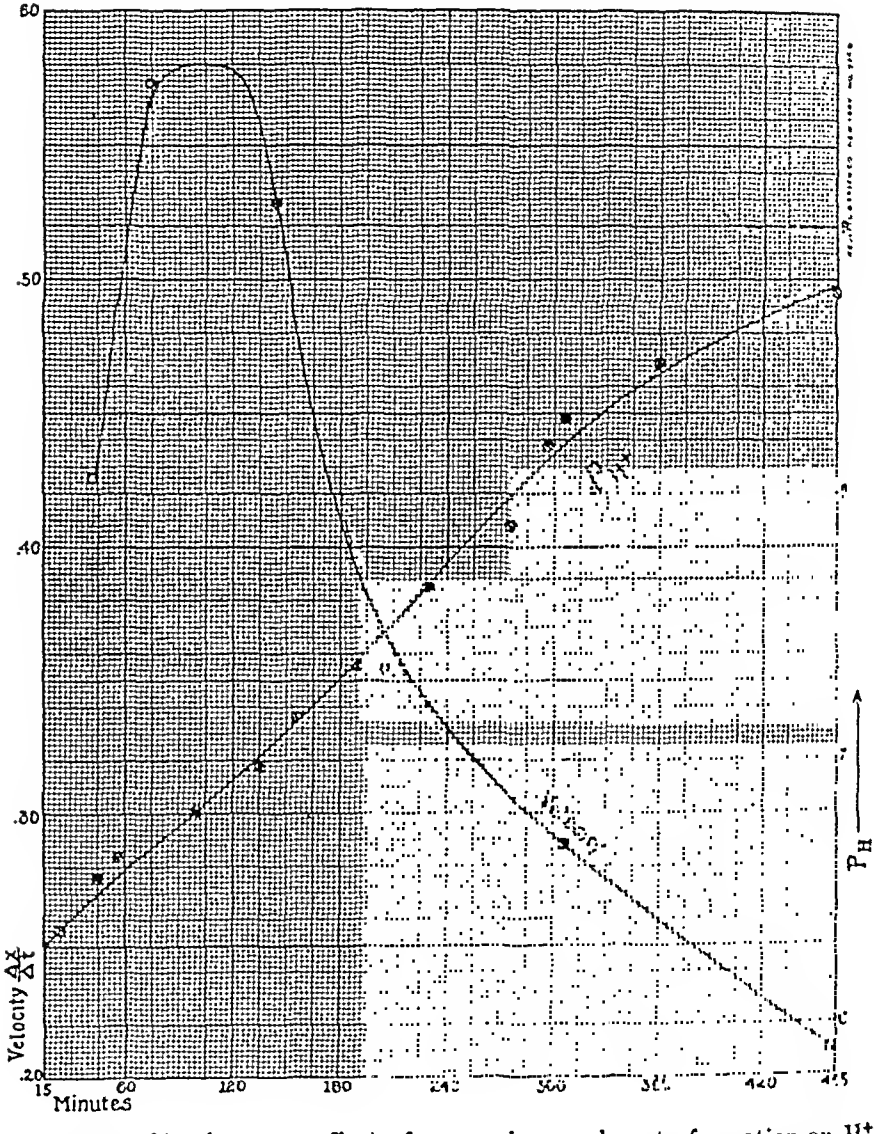


FIG. 2. Simultaneous effect of ammonium carbonate formation on H^+ concentration and reaction velocity. Reaction started in acid phosphate. Maximum velocity as P_H passes 6.8 (near neutral point). Results from Table III.

Constancy of reaction rate when change of H^+ concentration is prevented.

By addition of a mixture of primary and secondary phosphates in amounts large in proportion to the ammonia formed, the effect of the latter in changing the reaction of the solution towards the alkaline side can be almost completely excluded. Under these conditions the ammonia does not affect the velocity of the reaction.

TABLE IV.

Half-molecular phosphate concentration.

$\frac{M}{2}$ Na_2HPO_4	70 cc.	P_H at beginning of reaction	6.97
$\frac{M}{2}$ KH_2PO_4	30 cc.	P_H after 260 minutes	7.16
10 per cent urease	1 cc.	Temperature	20°
Urea	1 gram.		

t REACTION TIME	$\frac{N}{50}$ NH_3 FROM 10 CC. SOLUTION	x ($\frac{N}{1}$ NH_3 PER LITER)	MEAN VELOCITY $\frac{x}{t}$
	cc.		
7′	1.7	3.4	0.48
14′	3.4	6.8	0.48
25′	6.1	12.2	0.48
33′	7.6	15.2	0.46
55′	13.2	26.4	0.48
66′	14.35	28.7	0.44
85′	19.85	39.7	0.47

TABLE V.

Quarter-molecular phosphate concentration.

$\frac{M}{2}$ Na_2HPO_4	35 cc.	P_H at start of reaction	6 97
$\frac{M}{2}$ KH_2PO_4	15 cc.	P_H after 80 minutes	7.16
2 per cent urea	50 cc.	Temperature	20°
10 per cent urease	1 cc.		

t REACTION TIME	$\frac{N}{50}$ NH_3 FROM 10 CC. SOLUTION	x ($\frac{N}{1}$ NH_3 PER LITER)	MEAN VELOCITY $\frac{x}{t}$
	cc.		
9′	2.8	5.6	0.62
15.2′	4.9	9.8	0.64
25′	7.7	15.4	0.62
33′	9.7	19.4	0.59
53.3′	15.7	31.4	0.59
63′	18.5	37.0	0.59
79′	22.7	45.4	0.57
85′	24.55	49.1	0.58

From equation (4) of the preceding paper, viz., $\frac{dx}{dt} = E\frac{Km+nx}{K+x} = E\frac{231+x}{30+x}$, the velocity, $\frac{dx}{dt}$, would have fallen, when x has risen to 39.7, to 50 per cent of the initial rate, if nothing had been added to affect the alkalinity of the ammonium carbonate formed. For $x = 49.1$ (second table above) the velocity would be only 35 per cent of that at the start. In both the above experiments, on the other hand, where the H^+ concentration was kept constant in spite of the ammonium carbonate, the reaction rate remained practically constant from start to finish.

These results show that when the alkaline effect of the generated ammonium carbonate is obviated, the retarding effect on the reaction velocity is also prevented. The enzyme continues to decompose the same amount of urea per minute through a considerable part of the course of the reaction, and the velocity curve becomes a straight line.

These results confirm the view of E. F. and H. E. Armstrong, expressed in the following words: "In each successive interval of time the enzyme determines the hydrolysis of the same amount of hydrolyte; the observed departures from this rule may be attributed to the influence of the products of change."[6] This view, which applies a generalization of Duclaux to urease, was stated a year ago by the Armstrongs, but the above data are the first to prove it.

It should be mentioned further, that this rule does not hold through till the reaction has completely destroyed the hydrolyte (urea), but only as long as the latter is present in sufficient excess. As shown in the preceding paper, when the concentration of urea sinks too low to hold the proportion of uncombined enzyme down to a negligible value, the reaction rate begins to fall with the urea concentration.

Determination of initial velocities with variable H^+ concentration fixed by phosphate mixtures.

In order to rule out the effect on the enzyme of the increasing amounts of ammonium carbonate formed during the course of the

[6] *Proc. Roy. Soc.*, lxxxvi, p. 568, 1913.

reaction, the *initial* velocities of the reaction were determined in solutions that varied only in the proportions of primary and secondary phosphate, and the resultant H^+ concentration.

A series of solutions were made up as follows, all being alike, aside from the proportions of primary and secondary phosphate:

SOLUTION	VOLUME ADDED	CONCENTRATION IN FINAL MIXTURE (VOL. = 21 CC.).
	cc.	
Phosphate mixture, $\frac{M}{5}$	10	0.091 mol.
Urea, 2 per cent	10	0.91 per cent.
Urease, 2 per cent	1	0.091 per cent.

Ten cc. of each solution were placed in a stoppered aerating tube immediately after addition of the enzyme. After the solutions had stood thirty minutes at 20° the reaction was stopped with carbonate and the ammonia determined. The part of the solution not used for ammonia estimation served for H^+ determination.

TABLE VI.

Effect of H^+ concentration on initial velocity. Concentration of phosphates present, $\frac{M}{11}$.

NO.	$\frac{M}{5}$ Na_2HPO_4 IN 21 CC.	$\frac{M}{5}$ KH_2PO_4 IN 21 CC.	P_H	$\frac{N}{50}$ NH_3 FORMED IN 30′ IN 10 CC.	x ($\frac{N}{1}$ NH_3 PER LITER)	VELOCITY $= \frac{x}{30}$
	cc.	*cc.*		*cc.*		
1	0.2	9.8	5.3	2.6	5.2	0.17
2	0.25	9.75	5.7	3.4	6.8	0.23
3	1.0	9.0	6.1	5.7	11.4	0.38
4	5.0	5.0	6.97	9.1	18.2	0.61
5	7.0	3.0	7.38	8.9	17.8	0.59
6	8.0	2.0	7.50	8.6	17.2	0.57
7	9.0	1.0	8.04	7.25	14.5	0.48
8	9.8	0.2	8.80	4.85	9.7	0.32
9	10.0	0.0	9.24	3.35	6.7	0.22
10	10.0 + drop NaOH		9.36	3.0	6.0	0.20

The above data confirm those of the preceding experiment in placing the optimum H^+ concentration for the action of the urease at approximately the neutral point, $H^+ = 10^{-7}$ ($P_H = 7$).

Salt effect of phosphates in varying concentrations.

While neutral phosphate mixtures can maintain the enzyme action near its maximum rate by preventing the development of alkalinity, the phosphates also exert a retarding effect on the action. This action increases with the phosphate concentration, independent of the H^+ effect. For want of a more explicit name we have termed this the *salt effect* of the phosphates.

On reference to Tables IV and V, it will be noted that when the solution was made up with $\frac{M}{2}$ phosphate the velocity obtained was 0.48, while with phosphate in approximately $\frac{M}{4}$ concentration, other conditions being alike, the velocity was 0.62.

The data in the following tables, compared with that of the preceding, give further evidence of this effect, and also confirm the location of the optimum H^+ concentration near the neutral point. The details are the same as those of the preceding experiment, only the phosphate concentration being varied.

TABLE VII.

Effect of H^+ concentration on initial velocity. Concentration of phosphates present, $\frac{M}{16}$. Volume of each solution, 21 cc. Taken for NH_3 determination, 10 cc.

NO.	P_H			t DURATION OF REACTION	cc. $\frac{N}{50}$ NH_3 FORMED IN 10 cc.	x ($\frac{N}{1}$ NH_3 PER LITER)	VELOCITY $\frac{x}{t}$
	At start	At end	Average				
1	5.9	6.1	6.0	12′	2.9	5.8	0.48
2	6.8	7.0	6.9	12′	4.05	8.1	0.68
3	7.73	7.8	7.77	11′	3.55	7.1	0.64
4	8.04	8.2	8.1	11′	2.95	5.9	0.54
5	8.28	?	8.3	15′	3.55	7.1	0.47
6	8.28	8.9	8.6	15′	3.05	6.1	0.41
7	9.08	9.16	9.12	12′	1.90	3.8	0.32
8	9.16	9.24	9.20	12′	1.60	3.2	0.26

TABLE VIII.

Effect of H^+ concentration on initial velocity. Concentration of phosphates present, $\frac{M}{2T}$. Volume of each solution, 21 cc. Taken for NH_3 determination, 10 cc.

NO.	P_H			t DURATION OF REACTION	cc. $\frac{N}{16}$ NH_3 FORMED IN 10 cc.	x ($\frac{N}{1}$ NH_3 PER LITER)	VELOCITY $\frac{x}{t}$
	At start	At end	Average				
1	5.3	5.9	5.6	12′	2.4	4.8	0.40
2	6.3	6.5	6.4	12′	4.05	8.1	0.67
3	6.97	7.16	7.07	14′	4 7	9.4	0.67
4	7.38	7.73	7.56	14′	4.8	9.6	0.69
5	8.04	8.20	8.12	14′	4.2	8.4	0.60
6	8.79	8.95	8.87	14′	3.0	6.0	0.43
7	8.90	9.16	9.03	14′	2 3	4.6	0.33
8	9.97	?	10.00	14′	0.7	1.4	0.10

The results in the three above tables are presented by the curves of figure 3. They show that, in the concentrations of urea and phosphate present, the enzyme acts most rapidly in neutral solutions ($H^+ = 10^{-7}$). The curves are fairly flat at the top, so that very nearly optimum activity is attained when P_H is between 6.8 and 7.5 ($H^+ = 0.6 \times 10^{-7}$ to 3.2×10^{-7}).

The curves are markedly depressed at all H^+ points by the more concentrated phosphate solutions, the velocities at optimum H^+ being as follows:

With $\frac{M}{2T}$ phosphate..... 0.70
With $\frac{M}{16}$ phosphate..... 0.68
With $\frac{M}{1T}$ phosphate..... 0.61
With $\frac{M}{2}$ phosphate..... 0.48

Nature of the effect of varying salt concentrations on the action of the enzyme.

In order to analyze the effect of variations in neutral salt concentration we can utilize the equation developed in the preceding paper, $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$. If the salts interfere with the combination between enzyme and substrate, the constant c which represents the velocity of combination, will be depressed; if, on the other hand, the ability of the enzyme to decompose substrate

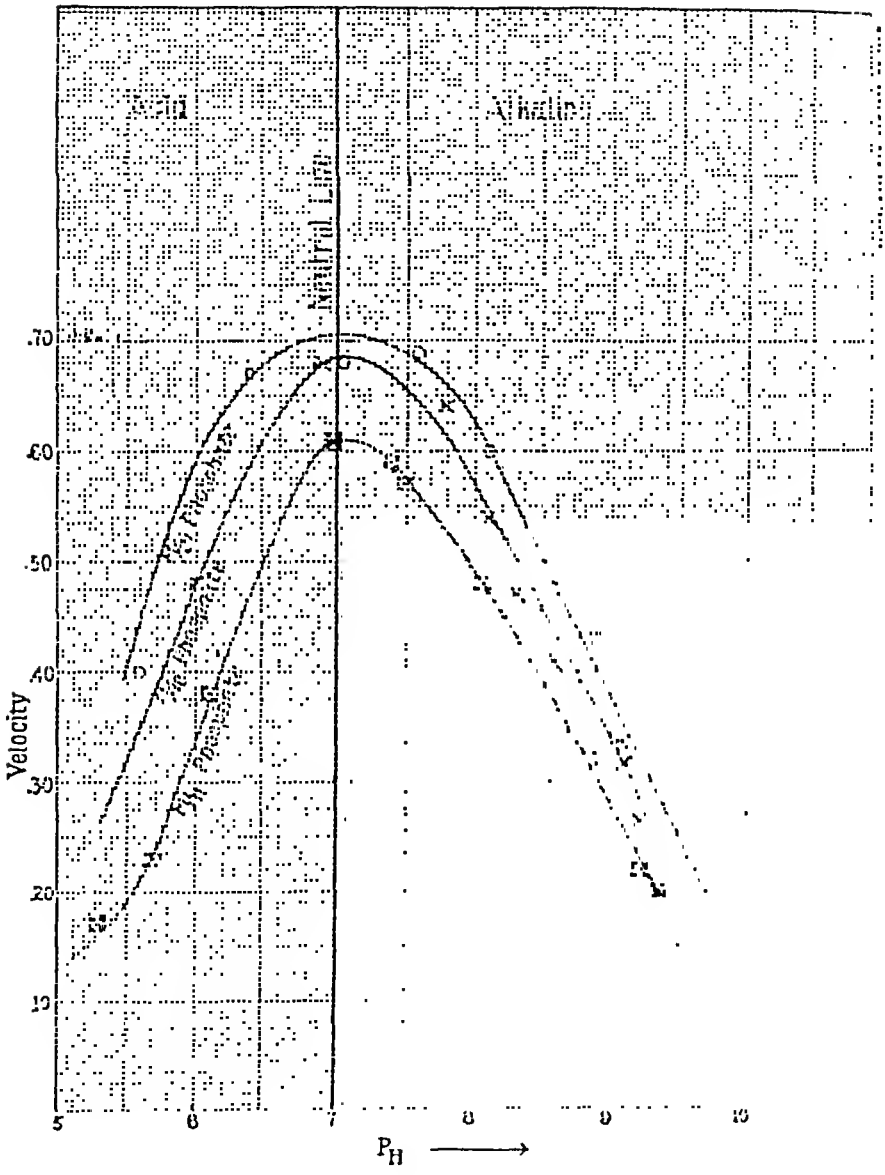


FIG. 3. Effect of H+ concentration. Urea = 0.91 per cent.

after combining with it is affected, the constant d, representing the velocity of the decomposition phase of the reaction, will be depressed. Complete inactivation of a portion of the enzyme will result in a proportional depression of both c and d.

The following series of experiments show that neutral phosphate depresses the combining constant c in direct proportion to the salt concentration. Sodium chloride also shows its effect almost entirely on c.

In each experiment 0.240 gram of urea with equimolecular amounts of KH_2PO_4 and K_2HPO_4 in the desired concentration, and in the molecular proportions 1:2 respectively, were dissolved in a 200 cc. flask, and diluted to 198 cc. The solution was brought to 20° in the bath, and 2.00 cc. of a 10 per cent solution of urease were added, the urease being the same preparation employed in the determination of c and d in the previous paper. The solution was mixed, and portions of 10 cc. were pipetted at once into 100 cc. test tubes which had already been brought to 20° in the bath. After varying intervals the tubes were removed from the bath and aerated into standard acid for a half minute (see technique of urine analysis, next paper). The enzyme action was then checked by addition of potassium carbonate, and the ammonia determined. The concentration of urea was 0.02 molecular, so the urea in 10 cc. would, if completely decomposed, yield 40 cc. of $\frac{N}{100}$ ammonia, equivalent to 40 cc. of $\frac{N}{1}$ ammonia from a liter. Therefore, in each series $a = 40$.

For determination of d (see preceding paper) the conditions were the same, except that the urea concentration was molecular (6 per cent). It was found that, under the conditions of all four series, this urea concentration is sufficiently high to be within the range where further increase is without effect on the velocity. Under these conditions $d = \frac{x}{Et} = \frac{x}{0.1t}$.

In the following tables, total phosphate concentration indicates the sum of the concentrations of K_2HPO_4 and KH_2PO_4, $\frac{M}{1}$ phosphate meaning $\frac{M}{3}$ $KH_2PO_4 + \frac{2M}{3}$ K_2HPO_4.

It is seen that, particularly in the more dilute phosphate solutions, the alkaline ammonium carbonate shows a small but appreciable effect in depressing the rate of reaction and consequently the value found for d, before the ammonia concentration x has

TABLE IX.

Effect of salt concentration on value of d in the equation

$$t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right). \qquad a = 2000.$$

$$\frac{1}{c}\log\frac{a}{a-x} \text{ is negligible.} \qquad d = \frac{x}{Et} = \frac{x}{0.1t}.$$

$\frac{M}{5}$ PHOSPHATE			$\frac{M}{2}$ PHOSPHATE			$\frac{M}{1}$ PHOSPHATE			$\frac{M}{5}$ PHOSPHATE + $\frac{4M}{5}$ NaCl.		
t	*x*	*d*	*t*	*x*	*d*	*t*	*x*	*d*	*t*	*x*	*d*
30′	16.0	5.34	20′	10.54	5.27	30′	12.4	4.12	35′	16.2	4.61
60′	29.0	4.84	60′	31.00	5.17	60′	23.4	3.90	90′	41.2	4.58
90′	44.6	4.95	137′	65.80	4.80	170′	62.2	3.66	140′	62.6	4.46
d for $x = 20$, 5.1			*d* for $x = 20$, 5.2			*d* for $x = 20$, 4.0			*d* for $x = 20$, 4.6		

TABLE X.

Effect of salt concentration on values of c in equation $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$.

$a = 40$ *in all cases.* $E = 0.1$. $c = \frac{d}{dEt-x}\log\frac{a}{a-x}$.

$\frac{M}{5}$ PHOSPHATE $d = 5.1$			$\frac{M}{2}$ PHOSPHATE $d = 5.1$			$\frac{M}{1}$ PHOSPHATE $d = 4.0$			$\frac{M}{5}$ PHOSPHATE + $\frac{4M}{5}$ NaCl $d = 4.6$		
t	*x*	*c*	*t*	*x*	*c*	*t*	*x*	*c*	*t*	*x*	*c*
15	5.4	0.0153	15	3.8	0.0058	30	4.4	0.00266	20	4.6	0.0053
30	10.4	0.0136	30	7.24	0.0058	45	7.0	0.00303	40	9.2	0.0057
45	15.0	0.0131	45	10.52	0.0055	60	9.1	0.00301	70	14.8	0.0055
60	19.2	0.0127	60	13.28	0.0052	95	13.2	0.00280	100	20.8	0.0058
80	24.8	0.0134	75	16.30	0.0052	130	15.5	0.00244	140	27.6	0.0063
100	29.8	0.0142	90	18.90	0.0050	170	20.2	0.00256	180	31.6	0.0067
121	33.0	0.0135	105	21.50	0.0052	210	24.5	0.00277	317	38.0	0.0058
135	36.2	0.0159	120	23.40	0.0052	310	31.1	0.00280	Average		0.0058
155	37.7	0.0153	135	25.36	0.0051	367	32.2	0.00247			
Average		0.0140	150	27.54	0.0053	Average		0.0027			
			165	29.58	0.0055						
			195	32.80	0.0057						
			225	34.54	0.0055						
			285	37.40	0.0056						
			345	38.76	0.0056						
			375	38.94	0.0053						
			Average		0.0053						

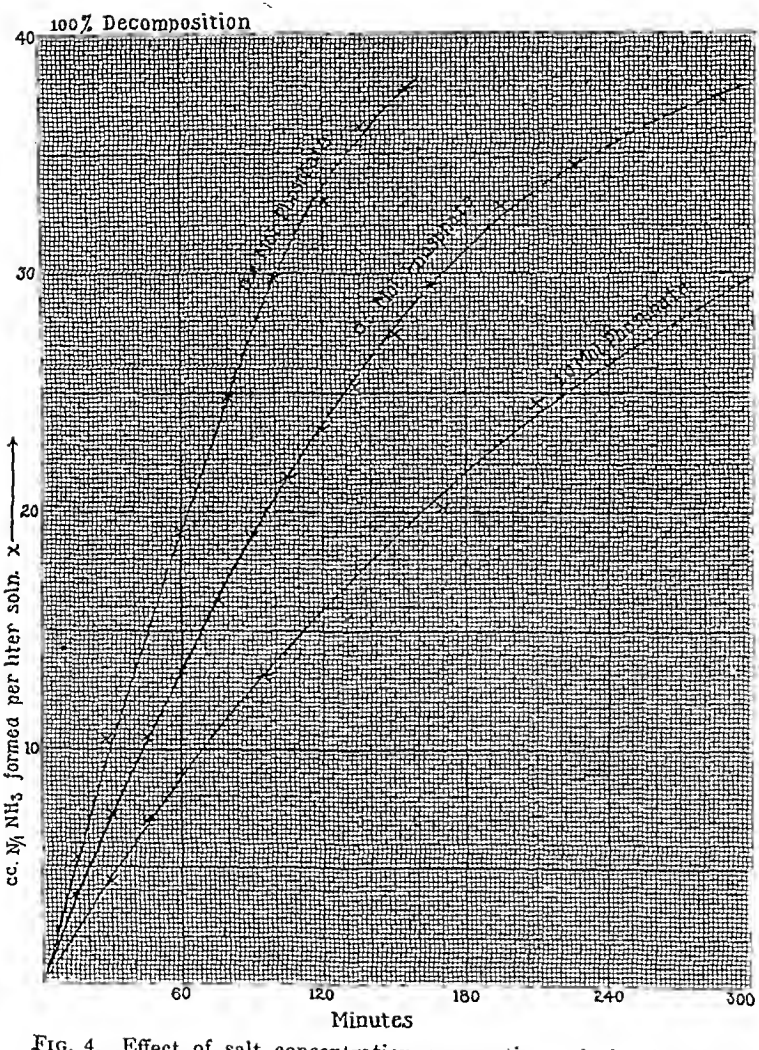


FIG. 4. Effect of salt concentration on reaction velocity in neutral solution.

TABLE XI.

Summary of results in Tables IX and X.

m MOLECULAR CONCENTRATION OF PHOSPHATE	*c* CONSTANT INDICATING VELOCITY OF COMBINATION OF ENZYME AND UREA	*d* CONSTANT INDICATING VELOCITY OF DECOMPOSITION OF ENZYME-UREA COMPOUND	*mc*
0.2	0.0140	5.1	0.0028
0.5	0.0053	5.17	0.0027
1.0	0.0027	4.0	0.0027

reached 40, its maximum value in the following series. For calculation of *c* in the latter we have chosen the interpolated value found for *d* when $x = 20$, when the reaction is half completed.

The results in the second and fourth columns show that the *combination* of enzyme and urea is *retarded at a rate directly proportional to the salt concentration.* On the other hand, the effect on the activity of the enzyme in decomposing urea, once it has combined with it is, as shown by the third column, slight. The drop to 4 at $\frac{M}{1}$ phosphate concentration is hardly due to specific effect, as it appears that any solution of such high osmotic pressure (even if the only solute is urea) depresses *d*.

Sodium chloride has an effect similar to that of phosphate, though not quite so marked.

Effect of glucose.

Glucose is known to retard the action of the sugar-splitting enzymes. The following results show that it has a similar effect.

TABLE XII.

DETERMINATION OF *d*.			DETERMINATION OF *c*. $a = 40$ $d = 4.4$.		
t	*x*	*d*	*t*	*x*	*c*
25	11.6	4.64	20	3.2	0.00285
80	16.4	4.10	40	5.6	0.00240
			75	11.0	0.00280
			130	15.0	0.00213
			176	22.0	0.00275
			210	24.6	0.00269
			270	29.4	0.00283
			Average		0.0027

on urease, and that the retardation affects, as in the case of the salt effect, the process of *combination* of enzyme with urea.

Glucose was added in $\frac{M}{1}$ concentration to a solution prepared as in the preceding experiments, and containing phosphate in $\frac{M}{2}$ concentration.

The glucose depresses c from 0.0053 to 0.0027, approximately one-half.

Effect of alcohol.

As Marshall has shown, ethyl alcohol in concentrations over 20 per cent markedly retards the action of urease. The following table shows that 30 volumes per cent alcohol (5.7 molecular) retards both phases of the enzyme action. The effect is quite unlike that of glucose and salts, as it is here chiefly d that is depressed. The phosphate concentration was $\frac{M}{5}$.

TABLE XIII.

DETERMINATION OF d. $a = 2000$.			DETERMINATION OF c.		
t	x	d	t	x	c
30	7.8	2.60	40	8.0	0 0105
60	14.6	2 46	60	11.8	0.0103
			105	19.5	0.0097
			160	28.2	0.0101
			200	32 4	0.0096
			Average		0.0100

d is depressed 50 per cent, from 5.1 to 2.5, while c is depressed only 29 per cent, from 0.0140 to 0.0100. The effect on d is what one might expect from the high osmotic concentration of the solution. The relatively slight effect on c indicates that alcohol, compared with glucose and the salts, interferes relatively little with the combination of enzyme and urea. The effect of the 5.7 molecular alcohol solution is only a fraction that of a molecular glucose or neutral phosphate solution.

Nature of the effect of H+ concentration changes on enzyme action.

The effect of H+ changes on the constants c and d was studied in the same manner as was the effect of salts, alcohol, and glucose. The phosphate concentration in all the following experiments was half-molecular, the proportions of primary and secondary phosphate being varied in order to produce the desired changes in the H+ concentration. The details of the experiments were the same as in the preceding.

TABLE XIV.

Acid solution. $P_H = 5.88$ *at start,* 5.90 *at end of experiment.*

DETERMINATION OF d. $a = 2000$.			DETERMINATION OF c. $a = 40$.		
t	x	d	t	x	c
50	17.0	3.4	50	1.8	0.000432
70	25.2	3.6	70	2.2	0.000374
100	33.2	3.3	110	3.7	0.000412
160	56.4	3.5	170	5.1	0.000370
			360	11.6	0.000440
Average		3.45	640	16.8	0.000385
			Average		0.00040

TABLE XV.

Slightly alkaline solution. $P_H = 7.65$ *at start,* 7.82 *at end of experiment.*

DETERMINATION OF d. $a = 2000$.			DETERMINATION OF c. $a = 40$. $d = 4.6$.		
t	x	d	t	x	c
30	14.2	4.72	45	16.2	0.023
70	32.2	4.60	65	22.0	0.020
100	42.8	4.28	115	36.0	0.027
			137	38.4	0.026
			Average		0.024

The value of d at the H+ concentration of Table 16 is extremely sensitive to slight increase in alkalinity, which the ammonium carbonate causes in this already alkaline solution despite the phosphate. Consequently a different value of d has to be taken for each value of x. The values obtained for d in the left half of

TABLE XVI.

Markedly alkaline solution. $P_H = 8.4$ *at start, 8.7 at end of experiment.*

DETERMINATION OF d $a = 2000$. (Because of increase in P_H with x, d decreases as x increases.)			DETERMINATION OF c. $a = 40$.			
t	x	d	t	x	d (corresponding to x)	c
30	9.0	3.00	50	13.0	3.14	0.22
60	16.2	2.70	70	17.0	2.91	0.35
110	25.2	2.29	110	23.6	2.66	0.28
180	34.8	1.93	150	29.0	2.17	0.29
			190	33.4	1.98	0.31
			Average			0.29

the table were plotted on coördinate paper as ordinates, with values of x as abscissae. The nearly linear curve gave graphically the proper values of d for each x in the lower right table.

TABLE XVII.

Summary of results showing effect of H^+ *concentration on* c *and* d *of equation*

$$t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right).$$

REACTION	P_H	$H^+ 10^7$	c	d	LOG $c \times 10^4$	LOG d
Acid	5.85–5.90	12–14	0.00038	3.45	0.58	0.54
Nearly neutral	6.8*	1.6	0.00530	5.17	1.72	0.71
Alkaline	7.65–7.82 Av. = 7.73	0.54–0.25	0.02400	4.6	2.38	0.66
Markedly alkaline	8.4–8.7 Av. = 8.55	0.04–0.02	0.29000	3.0–1.9	3.46	0.41

* Table V, preceding paper.

The relations are shown more clearly in figures 6 and 7. It will be seen that *d has its optimum at the neutral point,* but *c increases in direct proportion to the* $(OH)'$ *concentration* (inversely as H^+) throughout the entire range of the experiments (see linear curve of c, figure 7).

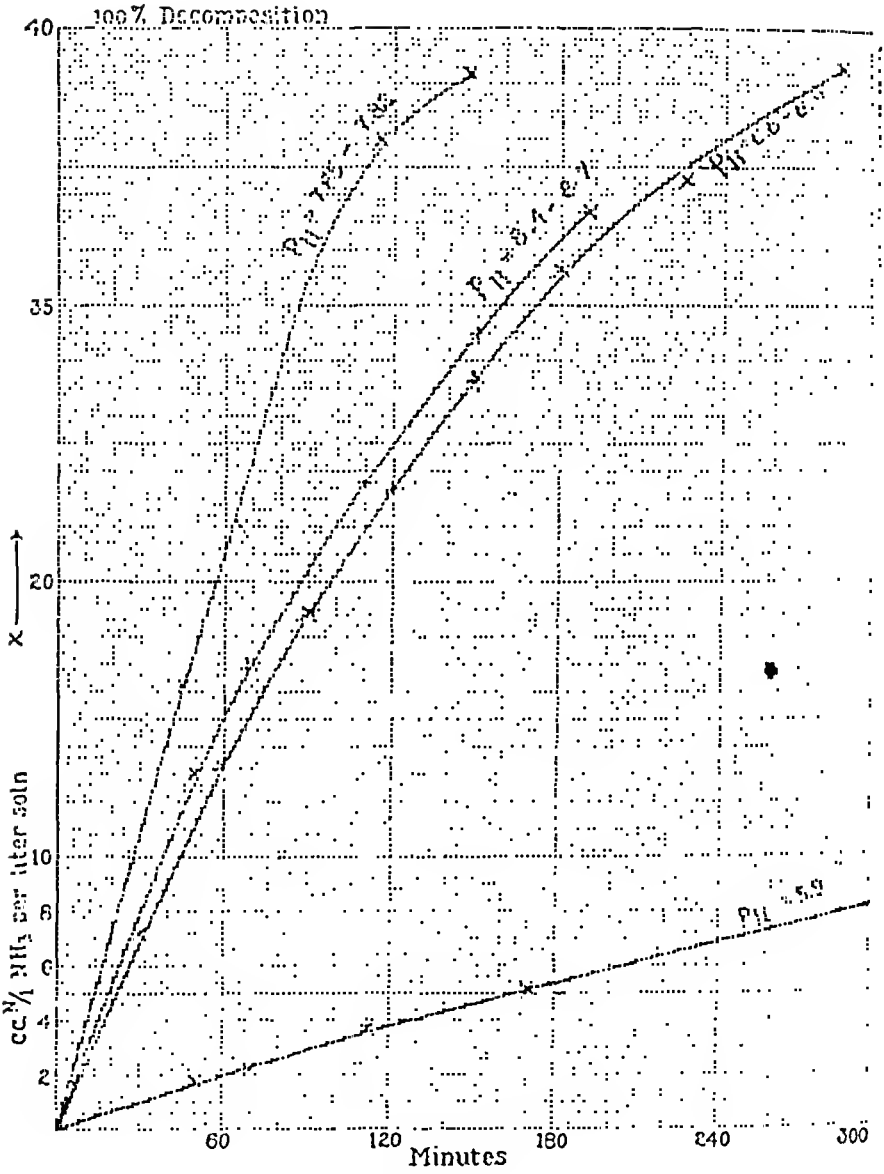


FIG. 5. Effect of H+ concentration on course of reaction in 0.12 per cent urea (a = 40).

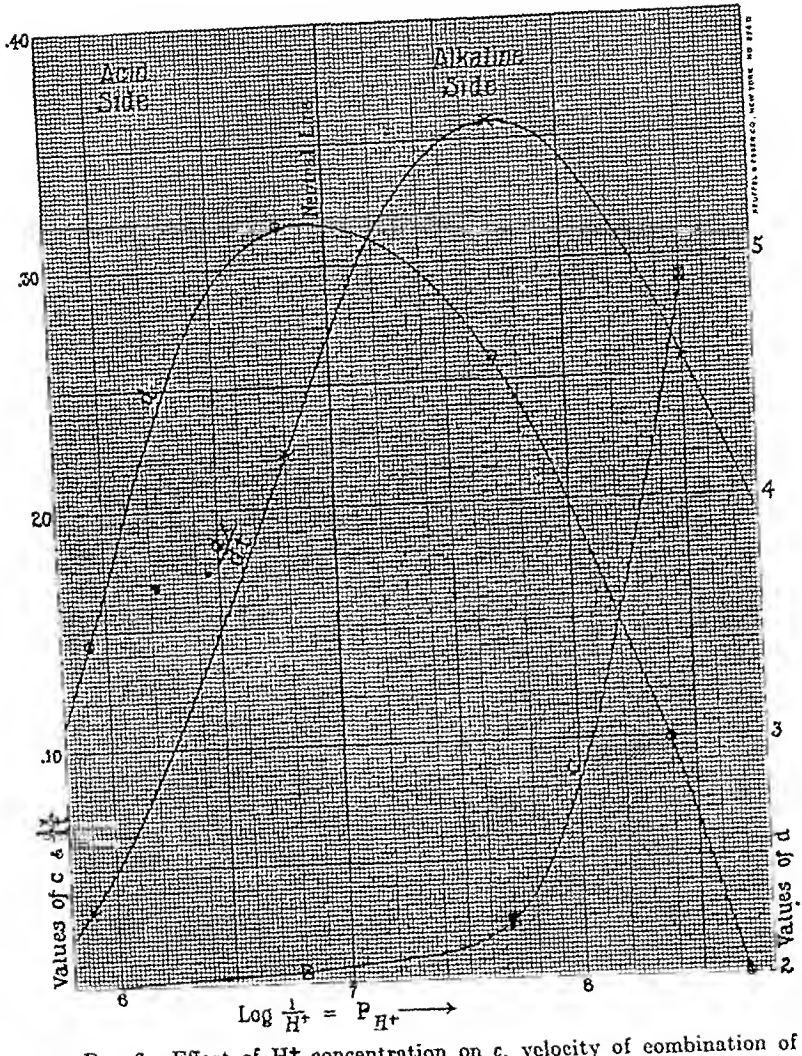


FIG. 6. Effect of H^+ concentration on c, velocity of combination of enzyme and urea; d, velocity at which enzyme splits off urea combined with it; $\frac{dx}{dt}$, resultant velocity of entire reaction.

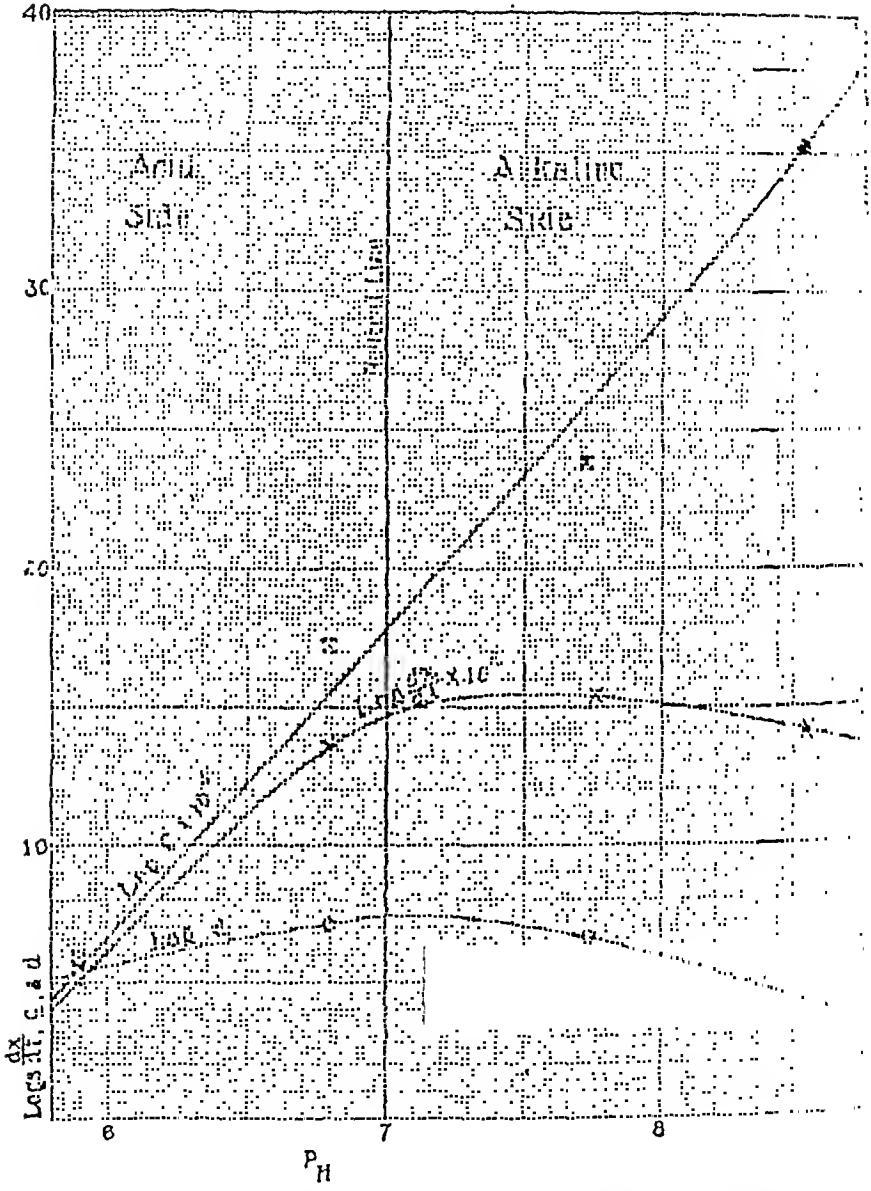


FIG. 7. Effect of H+ concentration on *c*, velocity of combination of enzyme and urea, points ⊠. *d*, velocity at which enzyme splits off urea combined with it, points ⊙. $\frac{dx}{dt}$, resultant velocity of entire reaction, points ×.

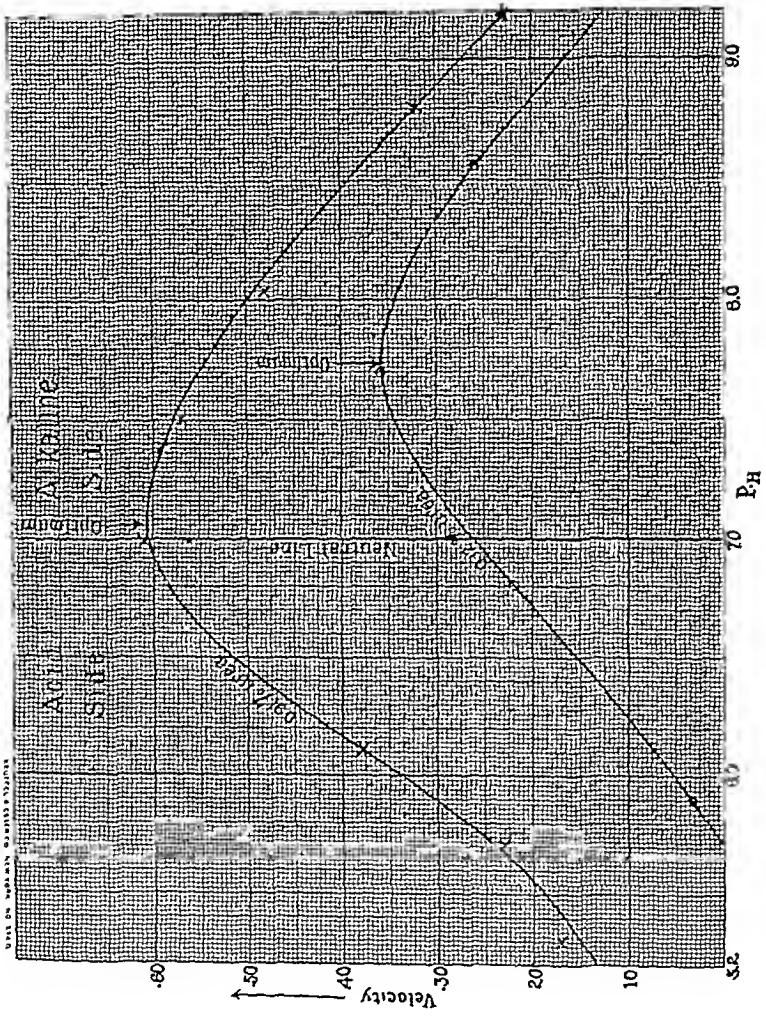


FIG. 8. Shifting of optimum H^+ to alkaline side by decreasing urea concentration. Phenomenon due to different effects of H^+ changes on c and d, respectively, in equation $l = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$.

Theoretical consideration of the effect of H^+ ion concentration and of inhibiting substances and products.

1. *Substances which retard combination between enzyme and substrate.* Returning to our original basic formula for the time required for the complete cycle of enzyme action (p. 149, preceding paper), we have

$$\theta = \frac{1}{cu} + \frac{1}{d} = \frac{1}{c(a-x)} + \frac{1}{d} \tag{1}$$

In case an inhibiting substance interferes with the combination, as do salts, glucose, and H^+ ions, the retardation being proportional to the concentration of inhibiting substance, we have

$$\theta = \frac{1+is}{c(a-x)} + \frac{1}{d} \tag{2}$$

s representing the concentration of inhibiting substance, i the constant expressing the intensity of its inhibiting effect. From the above we have, by the same steps used in deriving the equation, $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$ the following modification:

$$t = \frac{1}{E}\left(\frac{1+is}{c}\log\frac{a}{a-x} + \frac{x}{d}\right) \tag{3}$$

If the product $i\,s$ is large in proportion to 1, this equation approximates to

$$t = \frac{1}{E}\left(\frac{is}{c}\log\frac{a}{a-x} + \frac{x}{d}\right) \tag{4}$$

Here the coefficient of $\left(\log\frac{a}{a-x}\right)$ varies in direct proportion to i and s. For a given substance, i being constant, the coefficient, varies as the concentration, s, of inhibiting substance. This is what we have actually observed in the case of phosphate and H^+ concentration. The values expressed as c in the preceding tables actually represent values of $\frac{c}{i\,s}$, if we take as the true value of c that value which it possesses in the absence of any inhibiting substance.

Concerning the manner in which salts, H^+ ions, etc., retard the combination between enzyme and substrate, the most plausible explanation is that the inhibiting substances form compounds in

equilibrium with the enzyme, so that the latter is divided between substrate and inhibiting substance in proportion to the concentration of each, in accordance with the law of mass action.

By a study of the action of inhibiting substances of different chemical nature, it should be possible to obtain data that will indicate the nature of the chemical group or groups by which different enzymes combine with their substrates. Thus, the effect of H^+ ions on urease indicates that the combining group, the "adductor" of Armstrong, is basic.

2. *Substances which retard the action of enzyme on substrate with which it has combined.* In this case our formula becomes

$$t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{1+is}{d}x\right) \tag{5}$$

The experimental study of this action has not gone far enough to justify a general discussion. The value of d, totally unlike that of c, is depressed by either acid or alkaline reaction, d being at its maximum only in nearly neutral solution. The values of d, however, as affected by H^+ concentration, follow a curve exactly like that of the *undissociated fraction of an amphoteric electrolyte* of nearly equal basicity and acidity (see Fig. 6).

3. *The products as inhibiting substances.* (*a*) *The products retard combination between enzyme and substrate.* In this case our basic equation (2) above becomes

$$\Theta = \frac{1+ix}{c\,(a-x)} + \frac{1}{d} \tag{6}$$

From this:

$$\frac{dx}{dt} = \frac{1}{\Theta} = \frac{cd\,(a-x)}{d\,(1+ix) + c\,(a-x)} \tag{7}$$

By integrating (7), we have:

$$t = \frac{1+ia}{c}\log\frac{a}{a-x} + \left(\frac{1}{d} - \frac{i}{c}\right)x,$$

or, taking into account the effect of enzyme concentration,

$$t = \frac{1}{E}\left[\frac{1+ia}{c}\log\frac{a}{a-x} + \left(\frac{1}{d} - \frac{i}{c}\right)x\right] \tag{8}$$

This is similar in form to the equation of Henri discussed in the preceding paper.

(b) *The products do not interfere with combination of enzyme and substrate, but retard, in proportion to their concentration, the hydrolytic action of the enzyme on its substrate.* In this case our basic formula becomes:

$$\theta = \frac{1}{c(a-x)} + \frac{1+ix}{d}x.$$

By the steps of the preceding section this gives:

$$dx\left(\frac{1}{c(a-x)} + \frac{1}{d} + \frac{ix}{d}\right) = dt$$

$$t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x+2ix^2}{d}\right) \tag{9}$$

Equation (9) has as yet only theoretical interest, as we are not aware of any enzyme action in which the products exert their effect solely on the enzyme's specific action, of which the velocity is represented by d in the above equation.

SUMMARY.

1. *Different effects of H^+ concentration on the two phases of enzyme action.* Changes in H^+ concentration have absolutely different and independent effects on the two successive reactions [(1) combination with substrate; (2) decomposition of combined substrate] by which the enzyme destroys urea. Throughout the range of experimental observation (P_H = 5.9 to 8.7) *the combining velocity,* represented by c in the equation $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$, *varies in inverse ratio to the H^+ concentration;* the more alkaline, or less acid, the solution, the more rapid is combination. The second reaction, on the other hand, the *decomposition by enzyme of the urea combined with it* (velocity represented by d in equation above), *is most rapid in neutral solution, and is retarded by either alkalinity or acidity.*

2. *Conditions altering the optimum H^+ concentration.* In the absence of substances, such as salts and glucose, retarding the combination of enzyme and urea, the combination is so quick that, with experimentally practicable urea concentrations, it consumes a hardly appreciable proportion of the total time, which

is almost entirely taken up by the slower decomposition reaction. The maximum rate of total enzyme action, of ammonia formation, under these conditions is consequently fixed by that of the decomposition reaction, and is found at almost exactly the H^+ concentration (the neutral point) giving the maximum value for *d*.

When, however, the combining reaction is retarded (by adding neutral salts and using dilute urea solutions, for example) it becomes an appreciable factor in the total time consumption, and the optimum H^+ concentration for the total enzyme action is shifted towards the alkaline side. The optimum for the resultant of the two reactions is, as it were, a compromise between their respective optima, each exerting its influence in inverse proportion to the velocity of the corresponding reaction.

The effect of H^+ concentration on invertase noted by Sörensen is explainable by the same principle, independent effect on the two separate phases of enzyme action.

3. *Nature of the effect of the product, ammonium carbonate, on the action of urease.* The effect of the generated ammonium carbonate in retarding the action of urease is due chiefly to the alkalinity of the carbonate. This is shown by the following facts: (1) The reaction velocity decreases in proportion to the increase in OH' concentration, not to the ammonium carbonate. The first portions of the latter cause a great fall in the H^+ concentration, and a corresponding fall in velocity. After the carbonate has depressed the H^+ concentration to about $10^{-9.3}$, further increase in carbonate has almost no further effect on the alkalinity. From this point on, it also has little effect on the velocity. (2) When a phosphate mixture prevents the ammonium carbonate formed from affecting the H^+ concentration, it also prevents the effect on the velocity.

It is probable that ammonium carbonate also has a certain depressing "salt" effect on the action of the enzyme, as do even neutral salts (see below). The chief influence, however, is that of the alkalinity.

4. *Effect of neutral salts and non-electrolytes.* Neutral salts (phosphates and sodium chloride) retard the enzyme action. Their effect, so long as the osmotic pressure does not exceed that of a 2-molecular non-electrolyte solution, is exerted entirely in retarding the *first phase* of the enzyme's action, viz., the combina-

tion of enzyme and urea. The decomposition of urea after it has combined with enzyme is not affected.

Neutral phosphate mixtures have, therefore, a double effect on the enzyme. They maintain the reaction rate by preventing the alkalinity that would otherwise develop as soon as ammonium carbonate is formed, and consequently they act as accelerators. On the other hand, they themselves depress the activity of the enzyme by retarding its combination with substrate. This effect is the greater the more dilute the urea. Salts consequently retard especially the decomposition of the last traces of urea. Urease, therefore, shows its highest activity in the presence of the lowest concentration of phosphates that will maintain approximate neutrality.

Glucose retards the enzyme action in the same manner as neutral salts. Alcohol in 30 per cent concentration (5.7 molecular) depresses both phases of the enzyme's action.

Both electrolytes and non-electrolytes in osmotic concentrations much exceeding 2-molecular retard the enzyme action in its *second phase*, viz., the decomposition of urea after it has combined with enzyme. The effect is evident from a depression of d in the above equation, d being the velocity constant of the decomposition phase of the action.

5. *Formulation of the effects of inhibiting substances and products.* The general mass action formula for enzyme action, $t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right)$, derived in the preceding paper, is here further developed to include the effects of inhibiting substances on enzyme action, and the effect of products which influence either phase of the action.

A PERMANENT PREPARATION OF UREASE, AND ITS USE IN THE DETERMINATION OF UREA.[1]

By DONALD D. VAN SLYKE and GLENN E. CULLEN

(*From the Laboratories of the Rockefeller Institute for Medical Research, New York.*)

(Received for publication, July 14, 1914.)

While working with Marshall's already well-known method for the determination of urea by means of soy bean urease,[2] we have found that the enzyme can easily be prepared in the form of a soluble and very active powder, which can be accurately standardized and maintains its activity for an apparently indefinite period. By utilizing the high activity of the preparation, and the laws of its action developed in the two preceding papers, we have been able without complicating the technique to reduce the time required for an analysis from several hours to a few minutes. This shortening of the time, and the use of a permanent, standardized enzyme, remove whatever drawbacks the urease may have had as compared with the best chemical reagents previously used for urea determination.

The method requires no heat and little apparatus. Urease acts as well in diabetic as in normal urines. Its action is so specific that even in as complicated a mixture as blood it attacks nothing but urea. Its action can now be made almost instantaneous. These advantages make the enzyme method unique for combined accuracy and convenience.

Preparation of solid urease.[3] The preparation is very simple. The method which at present appears the most practical is based

[1] Preliminary report, *Soc. Exp. Biol. and Med.*, Dec. 17, 1913.

[2] Marshall: This *Journal*, xiv, p. 283, 1913; xv, pp. 487 and 495, 1913.

[3] The Arlington Chemical Company, of Yonkers, N. Y., has successfully taken up the problem of preparing solid urease on a large scale, and it can now be obtained in any amount from this firm. We acknowledge the courtesy of the company in supplying us with a large part of the enzyme used in our work.

on the fact that when a water solution of the enzyme is poured into a *volume of acetone so large that the enzyme undergoes practically instant dehydration*, it is precipitated with very little loss of activity. The precipitate can be dried in vacuum, pulverized, and kept indefinitely. The active portion dissolves almost instantly in water. A relatively small amount of flocculent residue does not dissolve, but this does not interfere with the activity of the enzyme or the measurement of the solutions by pipettes. An entirely soluble preparation is obtained by reprecipitating the filtered solution of the first precipitate. The volume of acetone used in each precipitation should be at least ten times that of the water solution of enzyme precipitated.

Another method which yields at once a permanent and entirely soluble preparation consists in simply concentrating the water extract of the soy beans at room temperature over sulphuric acid in a vacuum of less than 1 mm. The concentration is quite rapid, and the residue is so dry that it can be readily pulverized.

The extract for both the above preparations is prepared by digesting 1 part of soy bean meal with 5 parts of water at room temperature, with occasional stirring, for an hour, and clearing the solution by filtration through paper pulp, or centrifugation. The use of acid in clearing the solution would result in a considerable loss of activity.

Chemical nature of the solid enzyme. Chemical investigation of the precipitated enzyme showed that it contains a few per cent of ash, the organic matter being about two-thirds protein and one-third carbohydrate, chiefly cane sugar. Three days' digestion with trypsin or papain did not markedly injure the activity of the enzyme, so that it appears doubtful whether the urease is of protein nature. The enzyme could doubtless be freed of inert matter to a much larger degree than is the case with the preparations used, but there is no apparent advantage to be gained by removal of either the sugar or the protein.

Preparation and properties of the enzyme solutions. The "10 per cent" solution used in the following methods is prepared by dissolving 1 part by weight of the solid enzyme in 10 of water. Complete solution is most easily obtained if only enough of the water is added at first to moisten the enzyme. The mixture is stirred into a paste, then the rest of the water is added in portions.

Dilute solutions (1 or 2 per cent) of enzyme which have been prepared by either the concentration or double precipitation methods are water-clear. The 10 per cent solution is opalescent, like nearly all concentrated protein solutions. If the enzyme has been prepared by only a single acetone precipitation a small proportion remains insoluble in water, and forms a flocculent suspension. This does not interfere with the handling of the solution, however, and its activity is fully as great as that from the entirely soluble preparations.

When preserved under toluol in water solution the enzyme after a time loses its activity, so that it is best, as a rule, to use solutions made up the day they are used. The solutions will, if kept at 0°, maintain their activity without great loss for weeks however, particularly if a little K_2HPO_4 has been added. The preparation at present made by the manufacturers has 5 per cent of phosphate added because of its effect in preserving the activity of the enzyme in solution.

Standardization of the enzyme. In our preliminary experimental work we determined the activity of our enzyme solutions by letting 1 cc. act on 5 cc. of 5 per cent urea for fifteen minutes at 20°. The action was checked at the exact end of that period by addition of potassium carbonate, and the ammonia determined by aeration as described below. The amount of ammonia formed is approximately, though not exactly, proportional to the enzyme concentration. The ammonia formation does not increase as rapidly as the enzyme concentration, because of the alkaline effect of the ammonium carbonate on the enzyme.

When the alkalinity of the ammonia is neutralized by an equimolecular mixture of primary and secondary phosphate, however, the reaction rate is exactly proportional to the concentration of active enzyme.

Absolute standardization. Under the following conditions the ammonia formed in fifteen minutes is, with enzyme concentrations inside ordinary limits, directly proportional to the enzyme concentration. A solution is used containing 43 grams of K_2HPO_4 ($\frac{1}{4}$ molecule), 34 grams of KH_2PO_4 ($\frac{1}{4}$ molecule), and 60 grams of urea (1 molecule) per liter. To 5 cc. of this solution, placed in tube *A* (Fig. 2) and brought to 20° in a bath, one adds 1 cc. of the enzyme solution, which should also be at 20°. After exactly

fifteen minutes, 6 or 7 grams of potassium carbonate are added, and the ammonia is aerated into standard acid (25 cc. of $\frac{N}{50}$ usually), using the technique described for urine analysis. The result is expressed in the number of cc. of $\frac{N}{50}$ acid neutralized. A fresh 10 per cent solution of enzyme should show an activity of about 40. Although more active preparations can be made, this suffices for the use of the enzyme in the analyses described below.

Enzyme test under conditions of urine analysis. The following test is somewhat simpler. It shows whether the enzyme is active enough for the purpose of the analysis, but not to what extent it exceeds the necessary activity. A solution is made containing 3 grams, accurately weighed, of pure urea per 100 cc. Using the special pipette described in the urine analysis, one measures into tube *A* (Fig. 2) 0.5 cc. of the above urea solution, 5 cc. of 0.6 per cent KH_2PO_4, and the amount of enzyme solution intended to be used in analysis (usually 1 cc. of 10 per cent enzyme). The reaction is allowed to run at room temperature (or 50° if desired) for the length of time allowed in analysis, and the ammonia is determined as described for urine analyses. It should neutralize 25 cc. of $\frac{N}{50}$ acid. If it falls slightly short, it is well to repeat the test, doubling the time interval, as some samples of urea are not 100 per cent pure, and the short figure may be the fault of the urea, not of the enzyme. If in the longer interval no more ammonia is formed than in the shorter, the urea decomposition was complete in the shorter time, and the enzyme is sufficiently active.

Principles utilized in application of the enzyme. In the preceding papers it has been shown that:

1. The greater the *ratio*, enzyme : urea, the shorter the time required for complete decomposition of the urea. A given amount of enzyme decomposes per unit time the same *amount* of urea, regardless of the concentration of the latter over a very wide range (10 per cent to 0.08 per cent or less). The time required for complete decomposition of a sample of urea can therefore be decreased at will in exact proportion as one decreases the amount of urea in the sample or increases the amount of enzyme.

2. The ammonium carbonate formed retards by its alkalinity the action of the enzyme. The alkalinity and therefore the retarding effect can be prevented by the presence of acid phosphate.

3. Phosphates, like other salts, interfere with the combination of enzyme and urea which precedes hydrolysis of the latter. Consequently it is advisable to use the minimum amount of phosphate that will keep the reaction sufficiently near the neutral point.

4. Each 10° rise in temperature between 10° and 50° approximately doubles the rate of the reaction.

The effect of properly chosen phosphate addition is shown by the figure on the following page.

Determination of urea in human urine. Marshall has described two methods of utilizing urease for determination of urea in urine. One method consists in adding the enzyme solution to urine 20-fold diluted and, after the mixture has stood overnight, titrating the increased alkalinity with methyl orange as indicator.[4] The other method,[5] which he finds slightly more accurate, consists in adding the enzyme to 1 cc. of urine plus 10 cc. of water, letting the mixture stand overnight, with toluene, and driving off the ammonia by Folin's aeration method,[6] one hour's aeration being required.

We have found the process involving the Folin aeration best adapted for combined quickness and accuracy, and have been able to so fix conditions that the entire analysis can be finished in as short a time as ten minutes, although we ordinarily allow twenty-five or thirty. As an entire series of analyses can be run at once in this time, the method is very rapid.

One-half cc. of urine[7] is measured into the bottom of tube *A* (see Fig. 2). Exactly 5 cc. of a solution containing 6 grams of

[4] This *Journal*, xiv, p. 283, 1913.

[5] *Ibid.*, xv, p. 495, 1913.

[6] *Ibid.*, xv, p. 493, 1913.

[7] An Ostwald pipette is used, the stem of which is a heavy walled capillary tube of only 1 mm. bore. The pipette, which should deliver in about twenty seconds, is calibrated by weight for blow-out delivery, and permits measurement with an accuracy of 0.001 cc. The pipette is allowed to deliver with its tip against the lower part of the test tube wall until the bulb is emptied; the remainder of the contents is then blown out.

These pipettes, as well as the 100 cc. test tubes of special heavy glass, provided with inlet and outlet tubes for aeration, the block holder shown in the figure, and a brass aspirator pump suitable for the method, can be obtained from Emil Greiner, 45 Cliff Street, New York.

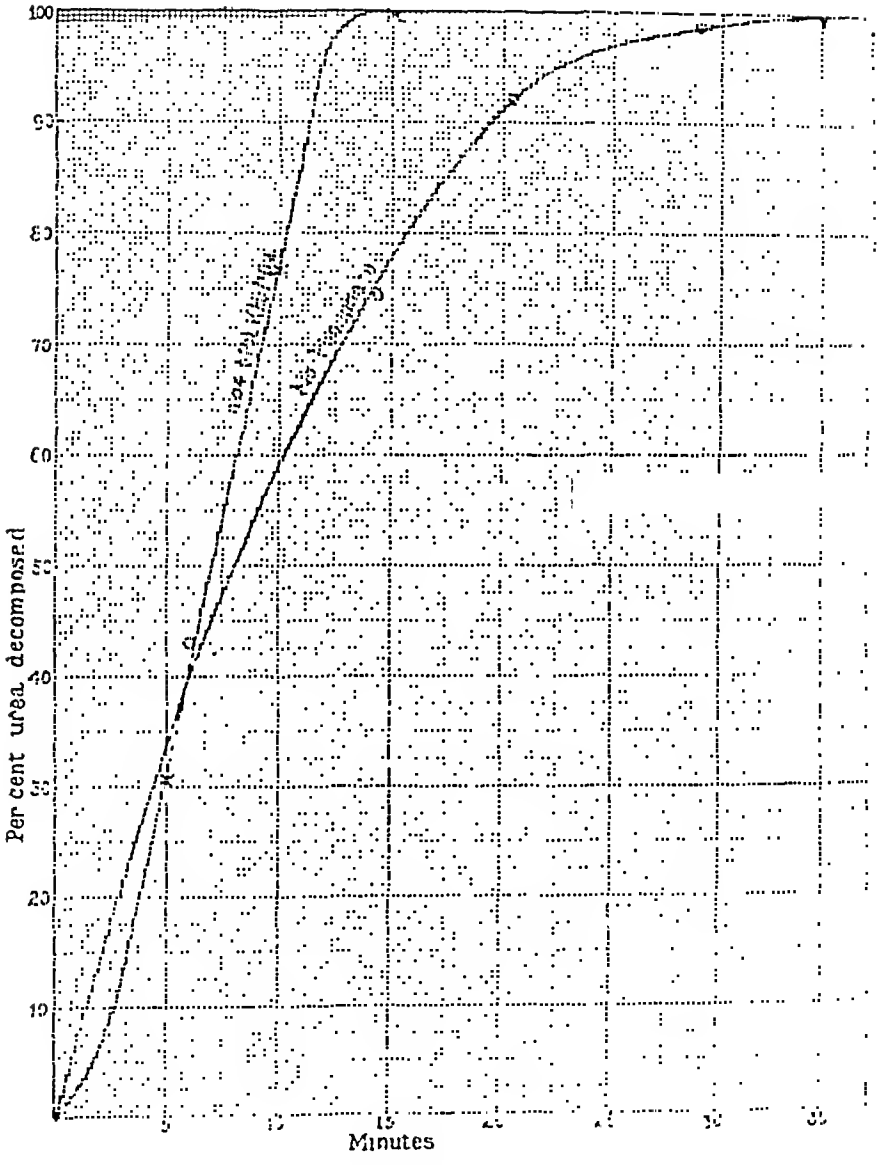


FIG. 1. Action of phosphate in accelerating reaction.

KH_2PO_4 per liter are then run in from a burette, and 1 cc., accurately measured, of a 10 per cent solution of urease[8] is added. The solutions in the tube are well mixed, 2 drops of caprylic alcohol to prevent subsequent foaming are added, and the stopper bearing the aerating tubes shown in the figure is put into place. Twenty

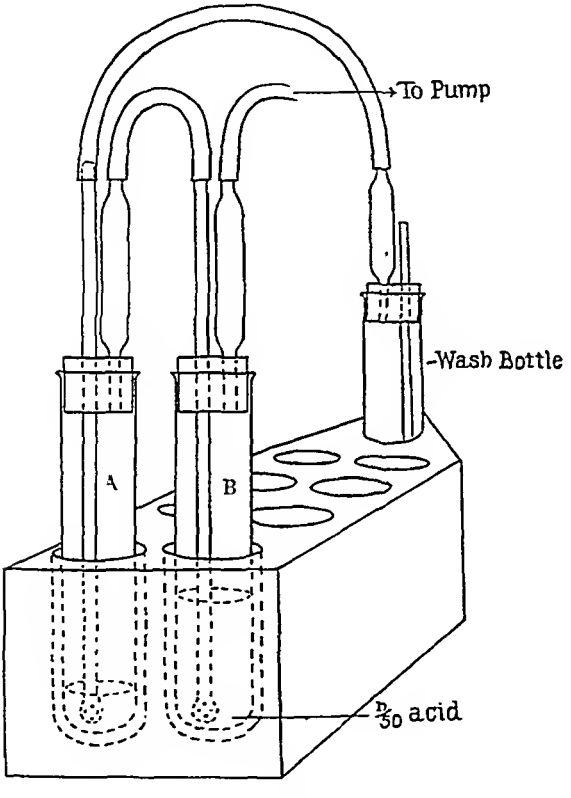


FIG. 2. Apparatus for determining urea content by means of urease.

minutes at a room temperature of 15°, or fifteen minutes at 20° or above, are allowed for complete decomposition of urea. No harm is done if the solutions are allowed to stand longer, but *the*

[8] The enzyme preparation used should be standardized as described above.

time must not be cut shorter unless more enzyme is used. While the enzyme is acting, one measures 25 cc. of $\frac{N}{50}$ hydrochloric or sulphuric acid into tube *B* and connects the tubes as shown in the figure. After the time for complete decomposition of urea has elapsed the air current is passed for a half minute in order to sweep over into *B* a small amount of ammonia which has escaped into the air space of *A* during the decomposition. *A* is now opened and 4 to 5 grams of potassium carbonate measured roughly from a spoon are poured in (in order to assure most rapid removal of ammonia by air current it is necessary to have the solution at least half saturated with carbonate). The air current is now passed rapidly through the tubes until all the ammonia has been driven over into the acid in *B*. The time required for this depends on the speed of the air current. With a rapid pump or house vacuum it is possible to aerate completely in five minutes; while a slow pump may require a half hour. The time required for complete aeration is determined for the particular vacuum used by trial, and a safe margin allowed in the determinations. When the aeration is finished the excess acid in *B* is titrated with $\frac{N}{50}$ NaOH.

The operations can be concisely summarized in the following diagrammatic form:

1. Measure into *A* $\begin{cases} \text{0.5 cc. urine.} \\ \text{5.0 cc. 0.6 per cent } KH_2PO_4. \\ \text{1.0 cc. 10 per cent urease.} \\ \text{2 drops caprylic alcohol.} \end{cases}$

Place stopper as shown in Fig. 2 and let stand fifteen minutes.

2. Meanwhile measure into *B* $\begin{cases} \text{25 cc. } \frac{N}{50} \text{ acid.} \\ \text{1 drop 1 per cent sodium alizarin sulphonate indicator.} \\ \text{1 drop caprylic alcohol.} \end{cases}$

3. (After 15 minutes standing) aerate one-half minute. Then open *A* and add 4 to 5 grams K_2CO_3.

4. Aerate all NH_3 from *A* over into *B*.

5. Titrate excess acid in *B* with $\frac{N}{50}$ NaOH.

6. Calculate: 0.056 × cc. $\frac{N}{50}$ acid = grams urea + ammonia nitrogen per 100 cc. urine.

In order to determine the *ammonia nitrogen* alone one measures 5 cc. of urine into *A*, adds the carbonate at once, and aerates as described above. The acid neutralized is multiplied in this case by the factor 0.0056, to give the per cent of ammonia nitrogen.

No extra time is required for the ammonia determination, as one merely aerates the extra pair of tubes in series with the same air current used for the ammonia + urea determination. As a matter of fact, one can conveniently run as many as eight pairs of tubes on the same air current, taking the precaution at the end of the aeration to disconnect the series in the middle first in order to prevent back suction.

Fixing the reaction time at will by regulating temperature and enzyme concentration. While the above conditions will probably be found most convenient for the usual analytical routine, they can be greatly varied without injury to the accuracy of the results. If the solutions are warmed to 50° during the reaction, the time can be reduced to three minutes, or the amount of enzyme can be cut from 0.1 gram to 0.02 gram, leaving the time at fifteen minutes. The general rule holds with mathematical exactness, that the time required for decomposition of a given amount of urea is, other conditions remaining the same, inversely proportional to the amount of enzyme present. Consequently one can economize on enzyme at the expense of time, or vice versa, as one prefers. By using a large amount of enzyme and warming to 50° one can effect complete decomposition of the amount of urea obtained in an ordinary urine analysis in as short a time as fifteen seconds.

Determination of urea in urines of animals. The method as above detailed is intended for urine containing not over 3 per cent of urea, which is about the maximum obtained in human urine. Urines from some other animals, such as the cat and dog, average much higher. Such are diluted sufficiently to reduce the urea below 3 per cent, then analyzed as above described.

Determination of urea in blood, spinal fluid, etc. The urease is particularly valuable in permitting a simple and accurate determination of urea in the blood, because its action is so specific that it attacks none of the other constituents, and the blood has to undergo no preliminary treatment for removal of the latter. This is true, at least, of normal blood and the pathological samples, showing urea up to 0.25 per cent, that have come to us for analysis.

The blood when drawn is mixed with 1 per cent its weight of solid potassium citrate to prevent clotting, and used for analysis within an hour. If it is analyzed fresh the ammonia content is negligible. Three cc. of blood or fluid are mixed with 3 cc. of the

0.6 per cent KH_2PO_4 solution and 1 cc. of the 10 per cent urease solution. Five drops of caprylic alcohol are added, and ten minutes are allowed for the enzyme to act. One measures 15 cc. of $\frac{N}{100}$ acid into tube *B*. From this point on the technique is the same as in the urine analysis, except that the titration is performed with $\frac{N}{100}$ NaOH instead of $\frac{N}{50}$. The calculation of urea is very simple, because each cc. of $\frac{N}{100}$ acid neutralized corresponds to 0.01 per cent of urea.

$$\text{Grams urea per 100 cc. blood} = 0.01 \times \text{cc. } \tfrac{N}{100} \text{ acid.}$$
$$\text{Urea nitrogen per 100 cc. blood} = 0.00466 \times \text{cc. } \tfrac{N}{100} \text{ acid.}$$

In case the blood should be one of the rare pathological samples containing more than 0.15 per cent of urea (the normal is 0.020 to 0.040), the entire 15 cc. of $\frac{N}{100}$ acid will be neutralized, and it may be necessary to repeat the analysis. If one sees the standard acid turn red, the aeration can be stopped and more acid run in. Otherwise the analysis is repeated, using 15 cc. of $\frac{N}{50}$ instead of $\frac{N}{100}$ acid.

The Folin colorimetric method can be used to determine the ammonia caught in tube *B*, only 1 cc. of blood and 0.3 cc. of enzyme solution being used. In our hands, however, the titration has given more consistent results.

Tissue extracts. Slight amounts of acids, even as weak as acetic, inactivate the enzyme. In case urea determinations are to be made on extracts of tissues which have been coagulated with the aid of acid, the latter must be neutralized with K_2HPO_4.

Technique of aeration. The technique is that of Folin, with some minor alterations to adapt it to the conditions. The tubes do not have to stand heat, and consequently can be made of very heavy, strong glass. We find the most desirable dimensions 26 x 200 mm., inner measurements. The tubes may be held for aeration in a holder made by boring holes 3 inches deep and $1\frac{3}{8}$ inches wide in a heavy block of wood. A convenient form is that shown in the figure. It holds four pairs of tubes, and has a hole at one end for a ninth tube which contains dilute acid and serves as a wash bottle to prevent the entrance of atmospheric ammonia into the system during aeration.

To prevent foaming, caprylic alcohol is the most efficient agent with which we are acquainted. With it one has no trouble at all,

in rapid aeration even of blood. In its absence one can use amyl alcohol, ethyl alcohol, or kerosene, although these do not guarantee the same freedom from annoyance and lost analyses. In view of the small amounts of caprylic alcohol required its use is not expensive.

It is advisable to use as air inlet a tube perforated with a number of pin holes not only in the standard acid (in test tube *B* of the figure), but also in the alkaline solution from which the ammonia is being driven. Experiments have shown that the ammonia is removed at a more rapid rate by the increased thoroughness of contact between air and solution which is gained by breaking the air stream up into small bubbles. The inlet tube for air must in every case *reach to the bottom of the solution.*

It is unnecessary to pass the air current through a second tube of standard acid. If one uses a *moderate current of air for the first minute* every particle of ammonia is absorbed in the first tube. Using violent currents from the start, we have been able to drive 1 to 2 per cent of the ammonia past the first tube, 98 to 99 per cent being absorbed even in these cases. The air is, of course, freed of atmospheric ammonia by passage through a wash bottle containing dilute acid before it enters the solutions under analysis.

The most convenient rubber tubing that we have found for the connections is the thick-walled, soft stethoscope tubing.

We have been surprised to find that the volume of the solution, from which ammonia is being removed, can be varied between 5 and 25 cc. without appreciably altering the time required to drive off the ammonia, so long as the solution is *at least half saturated with potassium carbonate.* If less than 1 gram of carbonate per 2 cc. of solution is added, however, more time is required to complete the aeration.

Use of the solid enzyme for Marshall's urea determination in urine by direct titration with methyl orange. The clinical method (direct titration with methyl orange of the increase in alkalinity of urine after action of urease) of Marshall[9] can be used with the permanent enzyme as well as with the extracts prepared according to Marshall's original directions. Instead of using 2 cc. of extract one adds 1 cc. of 10 per cent urease, and proceeds as directed by

[9] This *Journal*, xiv, p. 283, 1913.

Marshall, making a correction for the alkalinity of the enzyme solution. Five hours is sufficient for complete decomposition of the urea. We find, like Marshall, that this method gives with normal urines results practically identical with those by the aeration method. Diabetic urines, however, appear to contain substances which interfere with the accuracy of the titration. Although the end point does not appear different from that obtained with normal urines, the error may exceed 20 per cent.

EXPERIMENTAL.

Preparation of solid enzyme.

The following serve as examples of the results obtained by the different methods of preparing the solid enzyme.

One hundred grams of soy bean meal were mixed with 500 cc. of water and allowed to stand two hours, during which the mixture was stirred occasionally. The solution was then cleared by centrifugating. The following preparations were made.

a. Precipitation with 10 volumes of acetone. Fifty cc. of the extract were poured with stirring into 500 cc. of acetone. The precipitate which settled to the bottom was filtered at once and dried in a vacuum desiccator over sulphuric acid. Yield, 2 grams. The substance dissolved readily, except for a slight amount of flocculent residue.

Activity. One cc. of a 10 per cent solution of the preparation was allowed to act fifteen minutes at 20° on 5 cc. of 5 per cent urea. The ammonia formed neutralized 28.3 cc. of $\frac{N}{50}$ HCl.

b. Precipitation with 5 volumes of acetone. Fifty cc. of extract were poured into 250 cc. of acetone. The precipitate was treated as before, and the yield was the same. It left a much larger amount of insoluble residue when redissolved, however, and showed a decreased activity.

Activity, determined as in (*a*), 19.4 cc. of $\frac{N}{50}$ HCl.

c. Precipitation with 2 volumes of acetone. Again the yield was the same, but a large part of the precipitate did not redissolve in water, and the activity was reduced to 8.4.

d. Concentration to dryness at room temperature. Fifty cc. of the extract were placed in an evaporating dish and concentrated over sulphuric acid in a desiccator evacuated to 0.5 mm.

At the end of twenty-four hours the preparation was sufficiently dry to pulverize. Yield, 3.3 grams. The preparation redissolved completely in water.

Activity, determined as in (*a*), 26.4 cc. of $\frac{N}{50}$ HCl.

Analysis of pure urea solution.

The following determinations show the application of the method to amounts of urea, varying downwards from the maximum encountered in human urine. The conditions vary slightly from those finally selected as most desirable, in that the maximum amount of urea corresponded to that in only 0.3 cc. of urine, and the time for action of the enzyme was correspondingly shortened to ten minutes. Two cc. of urea solution were used in each analysis.

TABLE I.

CONCENTRATION OF UREA SOLUTION	$\frac{N}{50}$ HCl NEUTRALIZED	$\frac{N}{50}$ ACID CALCULATED
per cent	*cc.*	*cc.*
0.5	16.55	16.60
	16.62	
0.25	8.27	8.30
	8.28	
0.125	4.14	4.15
	4.18	
0.0625	2.06	2.07
	2.04	
0.025	0.85	0.83
	0.85	
0.0125	0.47	0.415
	0.43	

Urine analyses.

The determinations were run for ten minutes, 0.3 cc. of urine being taken, except in urines 1 and 4. Because these were unusually dilute, 0.6 cc. portions were used. For comparison the same urines were analyzed by Benedict's method.[10] In appreciation of Benedict's method we can say that, although it was

[10] Benedict: This *Journal*, viii, p. 405, 1910–11.

our first experience with it, every determination gave consistent results and none had to be repeated.

TABLE II.

URINE NO.	TAKEN	$\frac{N}{50}$ HCl	UREA + AMMONIA NITROGEN	UREA + AMMONIA NITROGEN BY BENEDICT'S METHOD
	cc.	*cc.*	*per cent*	*per cent*
1	0.6	3.60	0.168	0.171
		3.60	0.168	
		3.67	0.171	
2	0.3	11.88	1.111	1.076
		12.03	1.124	
		11.89	1.112	
3	0.3	12.79	1.195	1.192
		12.84	1.207	
		12.80	1.196	
4	0.6	11.62	0.543	0.546
		11.68	0.545	
		11.69	0.546	
5	0.3	13.50	1.261	1.284
		13.64	1.273	
		13.57	1.267	
6	0.3	6.12	0.572	0.563
		6.12	0.572	
		6.10	0.570	

Comparison of results by the aeration method and by direct titration in dilute urine with methyl orange.

The following data show that normal urines give the same results by both methods, but that diabetic urines give unreliable results when the titration is performed in the presence of the urine itself. The direct titration method was applied as directed by Marshall, the enzyme (1 cc. of 10 per cent) being added to 5 cc. of urine diluted with 100 cc. of water. After the mixture had stood overnight with toluene in a stoppered flask it was titrated with $\frac{N}{10}$ acid, using methyl orange as indicator. Controls were run with urine alone, and with enzyme alone, and the necessary corrections were made.

TABLE III.

Comparison of analyses of urines by aeration method and by direct titration with methyl orange.

SAMPLE NO.	NORMAL OR DIABETIC	GRAMS UREA NITROGEN PER 100 CC.		PERCENTAGE ERROR IN DIRECT TITRATION
		Aeration	Direct titration with methyl orange	
1	Normal	1.08	1.08	
2	Normal	1.33	1.34	
3	Normal	0.951	0.962	
4	Normal	0.412	0.412	
5	Normal	0.271	0.266	
6	Normal	0.294	0.292	
7	Normal	0.266	0.269	
0-3	Diabetic	0.384	0.308	−20
0-12	Diabetic	0.140	0.129	−8
P-4-15	Diabetic	0.803	0.829	+3
P-4-16	Diabetic	0.750	0.742	−1
P-1	Diabetic	0.216	0.258	+19
P-3	Diabetic	0.207	0.206	
P-12	Diabetic	0.301	0.356	+15
P-14	Diabetic	0.149	0.130	−13
P-16	Diabetic	0.149	0.122	−18
P-17	Diabetic	0.240	0.167	−30

Effect of phosphates on durability of enzyme in solution.

To one 10 cc. portion of soy bean extract 0.25 cc. of 20 per cent K_2HPO_4 was added; to a similar portion of the same extract 0.25 cc. of water. The extracts were covered with toluol in stoppered tubes and kept side by side at room temperature. The extract to which no phosphate had been added became more and more turbid and finally showed a heavy precipitate. The extract to which phosphate had been added was clearer at the end of the experiment than at the beginning.

TABLE IV.

DAYS STANDING	ACTIVITY OF EXTRACT WITHOUT K_2HPO_4	ACTIVITY OF EXTRACT WITH K_2HPO_4
0	17.0	17.0
5	15.4	16.6
8	14.8	16.1
15	11.9	15.2

The activities were determined by adding 1 cc. of extract to 5 cc. of 5 per cent urea, and determining the ammonia formed in fifteen minutes at 20°.

The preserving effect of phosphate is still more marked in the following experiment. The enzyme solution used was a 10 per cent solution of acetone-precipitated urease. The solutions, covered with toluene, were kept at room temperature, exposed to both direct and diffused sunlight.

TABLE V.

DAYS STANDING	10 CC. UREASE SOLUTION + 0.27 GRAMS (0.2 MOL.) KH_2PO_4 + 0.35 GRAMS (0.2 MOL.) K_2HPO_4	10 CC. UREASE SOLUTION + 0.27 GRAMS KH_2PO_4	10 CC. UREASE SOLUTION WITHOUT PHOSPHATE
	Activity $\frac{N}{50}$ HCl	Activity $\frac{N}{50}$ HCl	Activity $\frac{N}{50}$ HCl
	cc.	cc.	cc.
2	37.4	36.4	28.5
4	36.4	35.5	18.7
21	36.1	31.5	12.6
31	27.0	25.6	6.2

Effect of acid phosphate in accelerating enzyme action.

Two series of determinations were run, conditions being the same in both except that in one series KH_2PO_4 in 0.04 molecular concentration was present, while in the other none was added.

Series A. Each tube contained 5 cc. of 0.3 per cent urea (the amount of urea being the maximum usually present in urine analysis), 0.5 cc. of $\frac{M}{2}$ KH_2PO_4, and 1 cc. of 10 per cent urease solution.

TABLE VI.

SERIES A 0.04 MOL. K_2HPO_4			SERIES B NO PHOSPHATE		
Minutes	$\frac{N}{50}$ HCl	Per cent urea	Minutes	$\frac{N}{50}$ HCl	Per cent urea
	cc.			cc.	
5	7.7	30.8	7	10.78	43.1
10	19.2	76.8	14	18.55	74.2
15	24.95	99.7	21	23.00	92.0
20	24.98	99.9	28	24.65	98.6
25	24.98	99.9	35	24.95	99.8

Series B. The same as *A*, except that the phosphate solution is replaced by an equal volume of water.

After varying intervals the reaction was checked with potassium carbonate and the ammonia aerated. Temperature = 20°. Volume of each solution = 6.5 cc.

The effect of the phosphate is shown more clearly by comparison of curves of Fig. 1, which express the results above tabu-

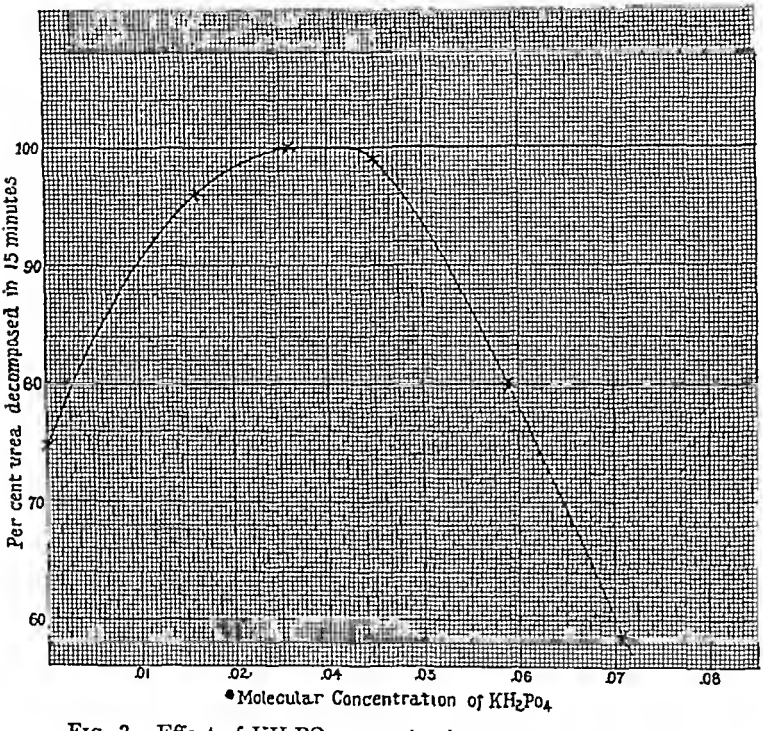


FIG. 3. Effect of KH_2PO_4 concentration on action of enzyme.

lated. That the phosphate curve is flatter at the start than after a little ammonia has been formed is due to the fact that KH_2PO_4 is too acid to allow maximum activity of the enzyme. Very little ammonia is required, however, to overcome this acidity, and as soon as 2 or 3 cc. of $\frac{N}{50}$ ammonia have been formed, the reaction assumes its maximum speed, which is maintained till decomposi-

tion is practically complete. In the absence of phosphate over twice the time is required for completion of the reaction.

That too much acid phosphate, on the other hand, retards the reaction is shown by the following table. The conditions were

TABLE VII.

Effect of varying concentration of KH_2PO_4 on enzyme action.

$\frac{M}{2}$ KH_2PO_4 ADDED	VOLUME OF SOLUTION	MOLECULAR CONCENTRATION OF KH_2PO_4 IN REACTING SOLUTION	$\frac{N}{50}$ NH_3 FORMED IN FIFTEEN MINUTES. (25 CC. = COMPLETE DECOMPOSITION)	UREA DECOMPOSED
cc.			*cc.*	*per cent*
0.0	6.0	0.000	18.70	74.8
0.2	6.2	0.016	24.00	96.0
0.4	6.4	0.031	24.96	99.8
0.6	6.6	0.045	24.75	99.0
0.8	6.8	0.059	20.00	80.0
1.0	7.0	0.071	14.62	58.5

similar to those of the above experiments, except that the time was constant at fifteen minutes, the phosphate being varied. To 5 cc. portions of 0.3 per cent urea varying amounts of $\frac{M}{2}$ KH_2PO_4 were added, and 1 cc. of 10 per cent enzyme.

The results are shown in Fig. 3.

METABOLIC CHANGES IN MUSCULAR TISSUE.

I. THE FATE OF AMINO-ACID MIXTURES.

By S. A. MATTHEWS and C. FERDINAND NELSON.

(From the Laboratories of Physiology and Physiological Chemistry of the University of Kansas, Lawrence.)

(Received for publication, August 4, 1914.)

The investigations of Folin and his coadjutors[1] have made clear many doubtful points in protein metabolism. It is only fair to give these investigators priority in establishing our present conception of the absorption and fate of protein digestion products (amino-acids) in the body. By their exact methods of blood and tissue analysis they have been able to trace the non-protein nitrogen compounds, formed in the intestine as the result of protein digestion, from the intestine into the blood of the general circulation and from the blood into the body tissues.

A little later D. D. Van Slyke,[2] by means of his nitrous acid method of determining amino-acids, was able to demonstrate the actual presence of these compounds as such in the blood and tissues, and that an increase in these products follows the ingestion of protein material.

Folin,[3] and later Fiske and Sumner,[4] have further been able to show that the general tissues of the body can, without intervention of any special organ, metabolize the non-protein nitrogen compounds which they found to increase in the blood and tissues after their absorption from the intestine, and that one of the products of this metabolism is urea. In fact, based upon their evidence, and the evidence we are about to introduce in this paper, there seems to be left no doubt but that the general metabolism of the body is carried out very largely by the tissues themselves.

[1] Folin and Denis: this *Journal*, xi, pp. 87 and 493, 1912.

[2] Van Slyke and Meyer: *ibid.*, xii, p. 399, 1912; xvi, p. 197, 1913.

[3] Folin and Denis: *ibid.*, xii, p. 141, 1912.

[4] Fiske and Sumner: *ibid.*, xviii, p. 285, 1914.

After protein substances have been hydrolyzed into amino-acids by the several proteolytic enzymes of the peptic, pancreatic, and intestinal juices, furnished by special aggregates of cells, constituted as glands, the further influence of specialized tissue upon amino-acid metabolism ceases; all subsequent metabolic processes being due to the general tissue cells acting as does unicellular life. This was suggested by one of us (Matthews) in a previous paper.[5]

The experimental evidence we have to present in this paper is the findings obtained from the analysis of the urine of dogs to which amino-acids were administered in such a manner as to insure their slow absorption and intimate contact with the tissues of the body, without any opportunity to come in contact with the cells of any organ of special function (such as the liver), which might in any way influence their metabolism.

Two sets of observations were taken on dogs, each set under a somewhat different setting of the experimental procedure, but essentially the same.

I. Observations upon Eck-fistula dogs after ligation of the hepatic, superior and inferior mesenteric arteries. About 85 per cent of the liver was removed.

II. Observations upon eviscerated dogs. All the viscera were removed except the kidneys and about 20–25 grams of the liver which could not be removed without injury to the vena cava.

In these experiments large female dogs were used which had fasted for thirty-six hours. The anesthetic was ether. A tracheal cannula was inserted in the usual manner and the animal decerebrated; otherwise the animals were prepared as already described (I and II).

Inasmuch as the tissues of an animal such as the dog are generally well saturated with nitrogen substances of one kind or another, we found it impossible to obtain any satisfactory measurements when working with tissues already thus loaded. To appreciate small variations it is necessary to calculate from a certain fixed point, preferably zero, if such a point is possible. We were able, at least in a measure, to approach such a point from which to compute our results. This we did in the following manner:

[5] Matthews and Miller: this *Journal*, xv, p. 87, 1913.

After an animal was prepared as already described a diuretic salt solution of the following composition was injected into one of the veins, usually the saphenous:

Sodium chloride	4 grams
Sodium sulphate	80 grams
Sodium citrate	27 grams
Calcium chloride	0.5 grams
Water to make	1000 cc.

During the experiment this solution was injected continuously, during the first hour at the rate of about 3 cc. per minute, and afterwards at the rate of about 2 cc. per minute, which was found sufficient to keep up a uniform flow of urine.

After the injection of about 200 cc. of this solution, which generally caused the excretion of about 225 cc. of urine, the urine was found almost ammonia-free, and contained only a small amount of nitrogen of any kind. A sample of this dilute urine of low nitrogen content was taken and the different nitrogen constituents estimated. This gave us a point, more or less approaching zero, from which to compute our results. After taking the first, or zero sample of urine, 10 grams of ereptone[6] dissolved in 50 cc. of warm water were injected into the muscles. The administration took about thirty minutes. First we attempted to inject the ereptone intravenously but found it readily eliminated by the kidneys largely unchanged. Apparently the elimination was so rapid that the tissues were not given time enough to exert any appreciable influence upon the substances injected. When injected into the muscles absorption took place quite readily but any urinary changes were long delayed. Evidently the ereptone was given an opportunity to come in contact with the tissues quite intimately and remain in such association for a considerable time. We attribute the results recorded in Tables I and II as due largely to the method of administration of the amino-acids by which they are brought into intimate contact with large surfaces of tissue for a considerable length of time. We consider the method here described for washing the tissue free from preformed nitrogen compound and the method of administering the

[6] Ereptone (Abderhalden) is a mixture of amino-acids. It contains no urea and only traces of NH_3. N = 12.75 per cent.

amino-acids as fundamental in determining the exact character of the metabolic changes which take place in muscular (elementary) tissue.

TABLE I.

Dogs fasted for 36 hours. Eck-fistula, hepatic superior and inferior mesenteric arteries ligated, and about 85 per cent of the liver removed.

	TOTAL N IN MGM. PER 1 CC. URINE	NH_3 N IN MGM. PER 1 CC. URINE	UREA N IN MGM. PER 1 CC. URINE
Dog I.			
Salt solution injected for 10 min.	24.3	1.9	22.0
Salt solution injected for 1 hour and 10 min.	2.0	0.7	1.0
Ten grams of creptone injected into the muscles. Time 30 min. Two hours after creptone injection	2.8	0.8	1.5
Dog II.			
Beginning of experiment		2.0	18.0
Salt solution injected for 1 hr. 30 min.		traces	0.3
Ereptone injected 30 min. Two hours 30 min. after creptone injection		0.3	0.6

TABLE II.

Eviscerated and decerebrated dogs. Otherwise animals prepared as in Table I.

	NH_3 N IN MGM. 100 CC. URINE	UREA N MGM. 100 CC. URINE
Dog I.		
Salt solution injected for 1 hour	30.0	315
Ereptone injected, 10 grams. Two hours after creptone injection	26.0	712
Three hours after creptone injection	47.0	630
Dog II.		
Salt solution injected for 1 hour	32.0	151
Ereptone 10 grams. Two hours after creptone	26.0	336
Three hours after creptone	39.0	320
Dog III.		
Salt solution injected 1 hour	6.3	210
Ereptone injected, 10 grams 1 hour after creptone	9.5	172
Two hours after creptone	18.0	159
Dog IV.		
Salt solution for 1 hour	10.8	118
Ereptone 10 grams, 1 hour after creptone	9.5	113
Two hours after creptone	10.5	127

As shown in Tables I and II the first nitrogen constituent of the urine to show a change after the injection of ereptone was the NH_3 which invariably appeared in the urine in increased amounts at the end of the first hour after the injection. We performed a large number of experiments on dogs and found this a never failing result.

The increase in urea which generally appeared from one to two hours later was not so constant, only being manifest in about 90 per cent of our experiments. In some cases the only result obtained was a marked increase of NH_3 accompanied by a decrease in urea (Dog III, Table II). In nearly all of our experiments in which we were able to keep the animal in good experimental condition (free from shock, etc.), and at the same time keep up a uniform flow of urine for five hours the result was an increased output of urea up to a maximum followed by a decrease after the fourth hour. It would seem from these results that the first action of the tissue upon amino-acid mixtures is the formation of NH_3 and later of the production of urea. It is a well established fact that urea is formed from ammonium compounds when perfused through the liver, and it is quite as likely that the muscular tissues of the body act upon such compounds in a like manner. Our results therefore will admit of the following interpretation only, namely, that the first action of the tissues of the body upon amino-acids is the formation of ammonia which in turn is converted into urea.

It might not be out of place in this connection to again cite, as corroborative evidence, more or less of a clinical nature, certain observations made by one of us (Matthews) upon Eck-fistula dogs.[7] These animals survived the operation for eighteen months and while unable to tolerate the ingestion of much meat in their diet enjoyed good health, when on a diet containing not more than 25 to 30 grams of meat per day. In these dogs the livers were unable to exercise any detoxicating influence upon the toxic substance formed in the intestines resulting from the digestion of meat. As always follows such operations the livers had undergone complete degeneration as shown by the histological findings. However, these dogs eliminated a normal amount of

[7] Matthews: this *Journal*, xv, p. 87, 1913.

nitrogen of which 80 to 85 per cent was in the form of urea. Of course this is not proof positive that the liver even after so complete degeneration may not still contain enzymes capable of forming urea from ammonia compounds. It is positive evidence, however, that the liver cannot exercise its usual protective influence against poisonous substances absorbed from the intestine when presented to it by way of the hepatic artery (one of these dogs was subjected to meat poisoning eight times during its lifetime after the operation). It might be argued that the blood supply to the liver by way of the hepatic artery is too small to permit it to act rapidly enough to do its usual chemical work and that therefore its powers are not impaired but only limited. Granting this, it is only fair to assume that its power to form urea from urea precursors would likewise be limited, and consequently the operation would cause a diminution in the output of urea, which was not the case.

SUMMARY AND CONCLUSIONS.

I. We have brought forward evidence of a positive nature showing that when amino-acid mixtures are injected into muscular tissue, these compounds are broken down and appear in the urine largely as ammonia and urea.

II. A method for determining the exact character of metabolic changes taking place in muscular tissue has been described.

III. We are at present working on the fate of individual amino-acids and other compounds of a protein nature when injected into muscular tissue in the manner above described.

THE SOLUBLE POLYSACCHARIDES OF LOWER FUNGI.

II. MYCOGALACTAN, A NEW POLYSACCHARIDE IN ASPERGILLUS NIGER.

By ARTHUR W. DOX and RAY E. NEIDIG.

From the Chemical Section of the Iowa Agricultural Experiment Station.

(Received for publication, August 8, 1914.)

In our first communication[1] on the polysaccharides of lower fungi it was shown that boiling water extracted from *Penicillium expansum* a new carbohydrate for which the name mycodextran was proposed. This substance was readily soluble in hot water and on cooling separated out in the form of a white paste. It showed a specific rotation of +251°, was unattacked by the ordinary amylolytic enzymes, and on acid hydrolysis yielded *d*-glucose.

Continuing our work on fungus polysaccharides, cultures of *Aspergillus niger* were extracted in the same manner. The cultures had not quite reached maturity although spores were already present. The hot extract on cooling gave a white slimy precipitate, possessing properties similar to those of the mycodextran previously prepared from *Penicillium expansum*. On cooling the filtrate from the mycodextran over night to 15° a transparent jelly formed. This was treated with an equal volume of alcohol, whereupon the jelly settled out leaving a clear amber colored supernatant liquid. The jelly was collected on a filter, washed several times with alcohol and finally with ether. A white powder resulted which was dried *in vacuo* over sulphuric acid and finally in an oven at 100°. As will be shown presently, this preparation is a galactan, for which the name *mycogalactan* is proposed.

Properties of Mycogalactan.

Solubility. The dry powder, prepared as above, first swells in cold water, then slowly dissolves, solution being facilitated by gently warming, No separation takes place on cooling to room

[1] This *Journal*, xviii, p. 167, 1914.

temperature, but when the solution is immersed in a freezing mixture a transparent jelly is formed. On adding alcohol, the jelly becomes so stiff that the beaker may be inverted without loss of its contents. In the presence of sodium hydroxide, the solution gelatinizes much less readily.

Iodine reaction. The aqueous solution gives a faint blue color with iodine. At present we are unable to state whether this is a property of myeogalactan or whether it is due to a trace of some other carbohydrate, like soluble starch or isolichenin. The absence of glucose was shown by the failure of the hydrolyzed solution to form an insoluble osazone when treated with the phenylhydrazine reagent.

Specific rotation. A 0.5 per cent solution in distilled water was prepared and examined polarimetrically in a 1 dm. tube at 20°. The average of five readings was +4.1°V.

$$[\alpha]_D^{20^\circ} = +284^\circ$$

Hydrolysis. A 1-gram sample was dissolved in 100 cc. of 2 per cent hydrochloric acid, and boiled under a reflux condenser for 10 hours. A slight formation of humus was observed. The solution was filtered, concentrated, nearly neutralized with sodium hydroxide and the volume made up to 100 cc. Two aliquots of 25 cc. were used for determining the reducing power by Allihn's method. The resulting cuprous oxide weighed 0.2355 and 0.2357 gm.; average, 0.2356 gm. corresponding to 0.1075 gm. glucose, or 0.8600 gm. glucose for the entire solution.

The remainder of the solution was polarized in a 2 dm. tube at 20°. Strong mutarotation was observed, which reached an equilibrium in two days, the final reading being +4.494°V. This value indicates galactose. Recalculating the reducing sugar above into galactose (0.898 gm. glucose is equivalent to 1 gm. galactose), we have 0.9577 gm. galactose in the solution. Using this value in calculating specific rotation, the result is

$$[\alpha]_D^{20^\circ} = +81.3^\circ \qquad [\alpha]_D^{20^\circ} \text{ for } d\text{-galactose} = +81.0^\circ$$

To confirm this finding of galactose, a sample of the polysaccharide was oxidized with nitric acid, according to the Official Methods, for the preparation of mucic acid. A good yield of mucic acid was

obtained which showed the correct melting point of 212°. A 0.25 gm. sample required 23.7 cc. of decinormal sodium hydroxide for neutralization; theory for mucic acid, 23.8 cc.

Ultimate analysis. A sample was oxidized in an ordinary combustion furnace with cupric oxide in the usual manner.

0.1026 gm. substance gave 0.1656 gm. CO_2 and 0.0590 gm. H_2O, leaving a residue of 0.0011 gm. or 1.07 per cent ash. From these figures the following percentages of carbon and hydrogen were calculated, on the ash-free basis:

	Found:	Calculated for $C_6H_{10}O_5$:
C	44.43	44.44
H	6.51	6.17

Mycogalactan has, therefore, the empirical formula common to most polysaccharides.

Mycogalactan, like mycodextran, appears to be a reserve carbohydrate, which is used by the fungus as a food supply as soon as the sucrose of the medium has become exhausted. As was stated at the outset, the cultures from which this preparation was made had not quite reached maturity and the medium showed very little, if any, evidence of autolysis. Subsequent attempts to prepare mycogalactan from more mature cultures that were beginning to show signs of autolysis met with failure. The alcoholic precipitate obtained from the aqueous extract of such cultures was curdy in appearance and consisted chiefly of one or more peptones giving the biuret reaction. However, from immature cultures a yield of 1 to 2 per cent was obtained, without contamination from protein decomposition products.

Galactans are known to occur in various organs, especially the seeds, of a large number of plants, both as pure galactans and mixed anhydrides with other sugars. They are an important constituent of marine algae, such as *Gelidium corneum* and *Fucus amylaceus*, of Chinese moss (*Sphaerococcus lichenoides*), and Irish moss (*Chondrus crispus*). Schardinger[2] reports the occurrence of galactan in bacterial slime, and Schützenberger[3] in yeast gum, but so far no galactan appears to have been isolated from molds. Our mycogalactan has a higher specific rotation than any other galactan previously studied.

[2] *Centralbl. f. Bakt.*, (2) viii, p. 144, 1902.

[3] *Compt. rend. de l'Acad. des Sci.*, lxxviii, pp. 493, 698, 1874.

BASAL METABOLISM AND CREATININE ELIMINATION.

WALTER W. PALMER, JAMES H. MEANS[1] AND JAMES L. GAMBLE.

(*From the Medical Service and the Chemical Laboratory of the Massachusetts General Hospital.*)

(Received for publication August 16, 1914.)

Of recent years the importance of a study of the basal metabolism both in normal and in pathological individuals has been clearly recognized. Benedict[2] and his associates have recently published figures for the basal metabolism in a large number of normal men and women. Benedict and Joslin[3] had previously made an exhaustive study of the same problem in diabetes. Coleman and Du Bois[4] have made important observations on the basal metabolism in typhoid fever and Du Bois recently in creatinism and exopthalmic goitre.[5]

These researches make it highly desirable to have some basis for comparing the basal metabolism of one individual with that of another. The comparison by means of body weight, that is, the calories per kilogram, has been felt for a long time to be unsatisfactory because large variations in the metabolism per kilo of body weight have been found to occur in individuals known to be normal. Also the greater the fat deposits the lower the metabolism per kilo, for fat is practically inert tissue. The same con-

[1] H. P. Walcott Fellow in Clinical Medicine, Harvard Medical School.

[2] Benedict, F. G., Emmes, L. E., Roth, P., and Smith, H. M.: The Basal Gaseous Metabolism of Normal Men and Women, this *Journal*, xviii, p. 139, 1914.

[3] Benedict, F. G., and Joslin, E. P.: Metabolism in Diabetes Mellitus, Carnegie Institution of Washington, Publication 136, 1910; also Metabolism in Severe Diabetes, Carnegie Institution of Washington, Publication 176, 1912.

[4] Coleman, W.: Diet and Metabolism in Typhoid Fever. Transactions 15th International Cong. Hyg. and Demog. Vol. ii, 602, 1912.

[5] Du Bois, E. F.: Paper on Calorimetric Observations in Patients with Diseases of the Ductless Glands, read at the meeting of the Amer. Assoc. for the Advancement of Clinical Research, Atlantic City, May, 1914.

dition might occur in an individual with unusually large bony structures.

Bergmann[6] first called attention to the relation between body surface and heat production, and later Rubner,[7] supported by experimental evidence, emphasized the importance of this theory. Rubner believed the basal requirement of any warm blooded animal to be approximately one thousand calories per square meter. Whether Rubner's law is invariably true or how accurately it holds for different individuals has not been established. The figures given by Benedict[8] make it appear that one thousand calories per square meter is too high. According to his results eight hundred calories is more nearly the correct value, although wide variations from this average occur.

Body surface as a means of comparison has certain marked disadvantages. If calculated from a formula such as Meeh's the errors may be very large in any except individuals of nearly average build. Actually to measure the surface in a large series of subjects would be an exceedingly laborious process.

Recently Benedict and Talbot[9] have called attention to the relation between pulse rate and metabolism. Murlin and Greer[10] suggest that pulse rate multiplied by pulse pressure may be of greater significance than pulse rate alone.

That the basal metabolism (and by basal metabolism is meant the minimum heat production that is consistent with normal cell life in an individual at complete muscular rest, without food, and surrounded by a temperature of 30° or more) is dependent upon the total mass of active protoplasm seems reasonable. The measurement of protoplasmic mass in a living animal might at once be regarded as impossible. Folin[11] in 1905 called attention to the constancy of the creatinine elimination in any one normal

[6] Bergmann and Leuckart: *Anatomische physiol. Uebersicht des Thierreichs*, Stuttgart, p. 272, 1852.

[7] Rubner, M.: *Zeitschr. f. Biol.*, xix, p. 545, 1883.

[8] Benedict, Emmes, Roth and Smith: *loc. cit.*

[9] Benedict, F. G., and Talbot, F. B.: Gaseous Metabolism of Infants, Carnegie Institution of Washington, Publication 201, 1914.

[10] Murlin, J. R., and Greer, J. R.: Relation of Heart Action to Respiratory Metabolism, *Amer. Journ. of Physiol.*, xxxiii, p. 253, 1914.

[11] Folin, O.: Laws Governing the Chemical Composition of Urine, *Amer. Journ. of Physiol.*, xiii, p. 66, 1905.

individual on a meat-free diet, and suggested that creatinine was a product of "endogenous" metabolism alone. The work of Folin and Denis[12] confirms this view. This important observation suggests the possibility that here we may have an index to the amount of protoplasm in the body, and that there may be some relation between basal metabolism and creatinine excretion.

The present investigation was undertaken in order to determine whether any such relationship between basal metabolism and creatinine elimination does exist. We wish merely to make a preliminary report on a short series of normal men and women between the ages of twenty and thirty.

EXPERIMENTAL PART.

We have calculated the basal metabolism by the method of indirect calorimetry. The gaseous exchange and the respiratory quotient were determined by means of Benedict's universal respiration apparatus.[13] The subjects were all in what Benedict calls the post-absorptive condition, that is at least twelve hours after food (before breakfast) and in complete muscular rest. Three ten to twelve minute periods were run on each subject and the figures for the three periods averaged. It is the averages which appear in the tables. In the case of a few of the subjects the basal metabolism was determined on two different days. When this is the case it is so noted.

The subjects were put on a diet without meat, fish or meat soups for a period of three days. A twenty-four hours specimen of urine was collected on the third day and the creatinine determined by Folin's method.[14] The basal metabolism determinations were usually made while the subjects were on the diet and when this was not the case a day or two before or after.

The results are given in three tables. Table I shows a series of observations on eight normal men, Table II on nine normal

[12] Folin, O., and Denis, W.: An Interpretation of Creatine and Creatinine in Relation to Animal Metabolism, this *Journal*, xvii, p. 493, 1914.

[13] Benediet, F. G.: Ein Universalrespirationsapparat, *Deutsch. Arch. f. klin. Med.*, evii, p. 160, 1912.

[14] Folin, O.: The Determination of Creatinine and Creatine in Urine, this *Journal*, xvii, p. 469, 1914.

women. In order to determine what variation in basal metabolism and creatinine elimination normally occurs from day to day, one subject, Dr. W. W. P. remained on the diet for six days and the basal metabolism and creatinine were determined daily. The results of this experiment are given in Table III. The extremes of basal metabolism in this series are from 1824 to 1780 calories per twenty-four hours, and of creatinine eliminated from 1960 to 1890 mgm. in twenty-four hours. In other words the results are sufficiently close to justify the method employed in the case of the other subjects, namely of making one or two determinations of the basal metabolism and creatinine.

TABLE I.

Observations on normal men.

SUBJECT	AGE	WEIGHT, KILOS	SURFACE AREA, SQUARE METERS, CALCULATED FROM MEEH'S FORMULA	CO_2 PER MIN., CC.	O_2 PER MIN., CC.	R. Q.	TOTAL CALORIES PER 24 HOURS	CALORIES PER KILO PER 24 HOURS	CALORIES PER SQUARE METER PER 24 HOURS	CREATININE, MGMS. PER 24 HOURS	CALORIES PER MGM. CREATININE	CREATININE PER KILO, MGM.	AVERAGE PULSE RATE
Dr. W. W. P....	32	93.9	2.541	250	284	0.88	2004	21.4	789	1980	1.01	21	62.0
Mr. H. L. H....	27	62.0	1.927	181	227	0.81	1574	25.4	817	1515	1.04	24	71.0
Dr. W. S. W....	25	73.8	2.164	194	240	0.81	1660	22.5	767	1860	0.89	25	64.0
Dr. L. W. H....	25	68.4	2.057	201	239	0.84	1671	24.5	813	1709	0.98	25	69.0
Dr. P. H. P....	27	77.2	2.179	204	230	0.89	1620	21.0	726	1690	0.96	22	50.5
Dr. J. H. M....	29	70.7	2.103	185	232	0.80	1599	22.6	760	1575	1.01	22	57.0
Dr. J. L. G.*..	30	68.1	2.051	200	241	0.83	1679	24.6	818	1759	0.95	26	55.0
Dr. L. H. N....	31	58.1	1.815	163	211	0.77	1452	25.0	787	1408	1.03	24	63.0

* This subject went on the special list for two three day periods about two weeks apart. Creatinine was determined at the end of each period and the figure given here the average. The basal metabolism was determined with each period and the figure given for that, the average.

Too few observations have been made to permit of definite conclusions. We should, however, like to call attention to certain relationships. The calories per milligram of creatinine are rather strikingly similar in each series, the ratio being higher in the women than in the men. The average number of calories per milligram of creatinine in twenty-four hours in the men is 0.98 while in the women it is 1.26. The average number of calories per kilo is about the same in both men and women, being approximately 23 in each, whereas the creatinine per kilo of body weight is much lower in the women, being on the average 18 as

TABLE II.

Observations on normal women.

SUBJECT	AGE	WEIGHT, KILOS	SURFACE AREA, SQUARE METERS, CALCULATED FROM MEEH'S FORMULA	CO_2 PER MIN., CC.	O_2 PER MIN., CC.	R. Q.	TOTAL CALORIES PER 24 HOURS	CALORIES PER KILO PER 24 HOURS	CALORIES PER SQUARE METER PER 24 HOURS	CREATININE IN GMS. PER 24 HOURS	CALORIES PER MGM. CREATININE	CREATININE PER KILO MGM.	AVERAGE PULSE RATE
Miss A. H. G.*.	23	52.6	1.688	166	205	0.81	1421	27.0	843	1175	1.21	22	70.5
Miss M. A. H.*.	21	57.9	1.841	174	204	0.85	1434	24.8	779	1153	1.24	20	75.0
Miss R. F. R.*..	20	58 5	1.854	164	210	0.78	1446	24.7	781	1143	1.26	20	73.5
Miss R. R.....	24	70 9	2.107	183	241	0.76	1648	23.2	780	1204	1.37	17	70.5
Miss H..	22	48.1	1.627	140	162	0.86	1143	23.8	705	900	1.24	19	78.5
Miss D. L... ..	21	76 0	2.205	180	214	0.84	1497	19.7	677	1275	1.17	17	73.0
Miss F. M. R...	20	77.7	2.240	193	237	0.82	1635	21.0	730	1390	1.18	18	80.5
Miss L. F. W...	21	79.8	2.280	173	214	0.81	1480	18.6	650	1110	1.33	14	60.0
Miss R. Rob....	23	67.5	2.039	172	207	0.83	1444	21.4	710	1090	1.32	16	66.5

* Two sets of three ten minute periods on respiration apparatus done several days apart, and results averaged.

TABLE III.

Series of observations on consecutive days on single subject (Dr. W. W. P.).

DATE	WEIGHT, KILOS	CO_2 CC. PER MIN.	O_2 CC. PER MIN.	R. Q.	TOTAL CALORIES PER 24 HOURS	CALORIES PER KILO PER 24 HOURS	CALORIES PER SQUARE METER PER 24 HOURS	CREATININE MGM. PER 24 HOURS	CALORIES PER MGM. CREATININE	CREATININE PER KILO MGM.	AVERAGE PULSE RATE
July											
21	93 8	228	252	0 90	1796	19.1	706	1940	0.93	21	60 0
22	93 9	229	253	0 90	1804	19 2	710	1890	0 95	20	59.5
23	93 8	226	253	0 89	1796	19 1	706	1940	0 93	21	61.0
24	94 0	225	260	0 87	1824	19 4	717	1920	0.95	20	60 5
25	93.8	222	251	0 88	1780	19 0	700	1960	0.91	21	61.5
26	93 1	215	255	0 84	1785	19 2	702	1900	0.94	20	63.5

against 24 in the men. It should be pointed out that the average, 28, of the men is greater than the average age, 22, of the women. With one exception (A. H. G.) the women may be considered as having proportionately greater fat deposits than the men. It is interesting that the women with apparently greater amounts of fat have higher ratios between basal metabolism and

creatinine excretion. Whether this is due to a relatively greater respiratory metabolism or low creatinine elimination is not clear from the few data available. The individuals on whom our observations were made were all young adults. That the same relation may not hold for infants, children and elderly individuals is quite possible. Further observations are being made on the relation between creatinine elimination and basal metabolism in subjects of varying ages and in disease.

The subjects of this research were chiefly nurses and physicians in the hospital. We wish to thank them for their hearty coöperation. Our thanks are also due to Miss A. H. Gardiner, Miss M. N. Marble and Miss Etta Mullen for their valuable assistance in carrying out the investigations.

OBSERVATIONS ON THE ISOLATION OF THE SUBSTANCE IN BUTTER FAT WHICH EXERTS A STIMULATING INFLUENCE ON GROWTH.[1]

By E. V. McCOLLUM and MARGUERITE DAVIS.

(From the Laboratory of Agricultural Chemistry of the Wisconsin Experiment Station.)

(Received for publication, August 19, 1914.)

In a previous paper[2] we described for the first time the behavior of rats fed for long periods on rations of purified food substances, but containing no fat.[3] Such rats may grow for as long as three to four months at a rate not far from normal. Sooner or later, however, all animals on such rations cease to grow, although they may maintain a fairly good state of nutrition for a considerable time after growth ceases. In our paper we pointed out the fact that the addition of the ether soluble portion of butter or of egg yolk to the diet after growth was completely suspended would induce a prompt resumption of growth. We reported at that time a reproduction and complete nutrition of the young on a diet of purified food substances with these fats added to the extent of about 5 per cent of the ration. A year previous to the appearance of our paper, Osborne, Mendel and Ferry[3] after observing apparently normal nutrition in rats on such diets free from fats, during a period of about sixty days, said:

McCollum has demonstrated that the phosphorus needed by an animal for phosphatide formation can be drawn from inorganic phosphates, and that phosphatides can be synthesized anew in the animal body. Rohmann asserts the possibility of lecithin synthesis in mice which were maintained into the second generation on lecithin-free food. Our own experiments

---

[1] Published with the permission of the Director of the Wisconsin Experiment Station.

[2] This *Journal*, xv, p. 167, 1913.

[3] Osborne, Mendel and Ferry had previously shown that normal growth can be made on such diets during sixty days (this *Journal*, xii, p. 81, 1912.)

point in the same direction with regard to the lipoids in general; and they give positive evidence of the dispensableness of true fats for growth.

Our own experience at that time was not in harmony with the idea that the lipoids in general were dispensable in the diet during growth. Since our paper was published Osborne and Mendel have verified our results by experiments using clear centrifugated butter fat, and also egg fat, and have extended our knowledge concerning the distribution of the growth promoting substances carried by fats, to commercial cod liver oil. They have likewise shown that almond oil does not behave like butter and egg fats in inducing a resumption of growth, but behaves like olive oil which we found to be inefficient in this respect.

We have now observed that the property of inducing a resumption of growth in rats which have grown as far as possible on a fat-free ration, can be conferred on olive oil by shaking the latter with a solution of the soaps prepared by completely saponifying butter fat in a non-aqueous system with potassium hydroxide, according to the method of Henriques.[4]

Forty cc. of butter fat obtained by melting butter at 45° and pipetting off the clear butter fat, were dissolved in 200 cc. of absolute alcohol and 200 cc. of petroleum ether, and 72 cc. of 15 per cent absolute alcoholic potassium hydroxide added. The mixture was agitated for a time in a closed bottle and allowed to stand at room temperature until saponification was complete. The soaps separate partially as a jelly. The contents of the bottle were spread in a thin layer on flat dishes and the solvent completely removed in a vacuum. The soaps thus prepared were light yellow in color and pulverized readily to a fine powder. This was placed in a large separatory funnel and 500 cc. of water and a drop of phenolphthalein added. The funnel was agitated and while the soaps dissolved dilute HCl was added just fast enough to discharge the pink color of the indicator. When the soaps were all dissolved 20 cc. of olive oil were added and the funnel agitated until a permanent emulsion was secured. About 500 cc. of ether were then added and after thorough agitation the separatory funnel was allowed to stand until the ether-olive oil layer separated sharply. This required at least twenty-four hours. The

[4] Henriques: *Zeitschr. f. angew. Chem.*, p. 721, 1895.

soap solution was drawn off and the ether-olive oil washed once with a liberal volume of water, NaCl being added to facilitate the ether separation. Adequate time for as complete a separation of water and ether as possible was allowed. The ether-olive oil layer was then separated and the ether evaporated in vacuum.

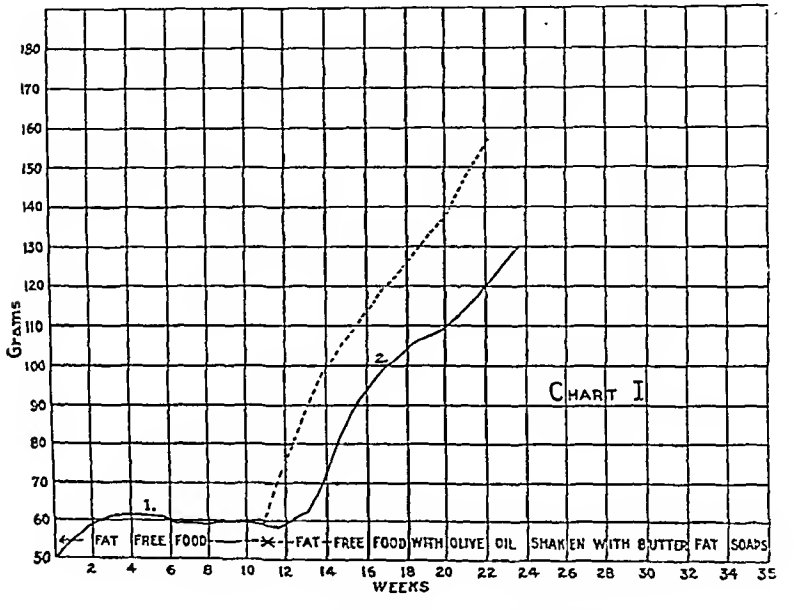


CHART I. (Male rat). Showing the effect of adding to the diet olive oil which had been shaken with a solution of soaps prepared from butter fat. This was an animal of low vitality as indicated by its inability to make any growth on the fat-free diet. The addition of olive oil thus treated, to the extent of about 3 per cent of the diet, induced a prompt resumption of growth at about normal rate. At this date there is no sign of failure of growth.

Dotted line shows the normal curve of growth.

The olive oil was warmed to 45°C. and again placed in the desiccator in vacuum for a few hours. This olive oil was fed in amount equivalent to about 3 per cent of the diet.

The accompanying charts indicate clearly the stimulating effect of the olive oil thus treated on growth as compared with the same oil without such treatment and seem to leave no room for doubt

that the growth promoting substance was transferred from the butter fat to the olive oil by the procedure described.

In an extensive experience with rations made up of purified casein, dextrin and salt mixtures from reagents[5] we have observed a marked difference in the ability of individual rats to grow on

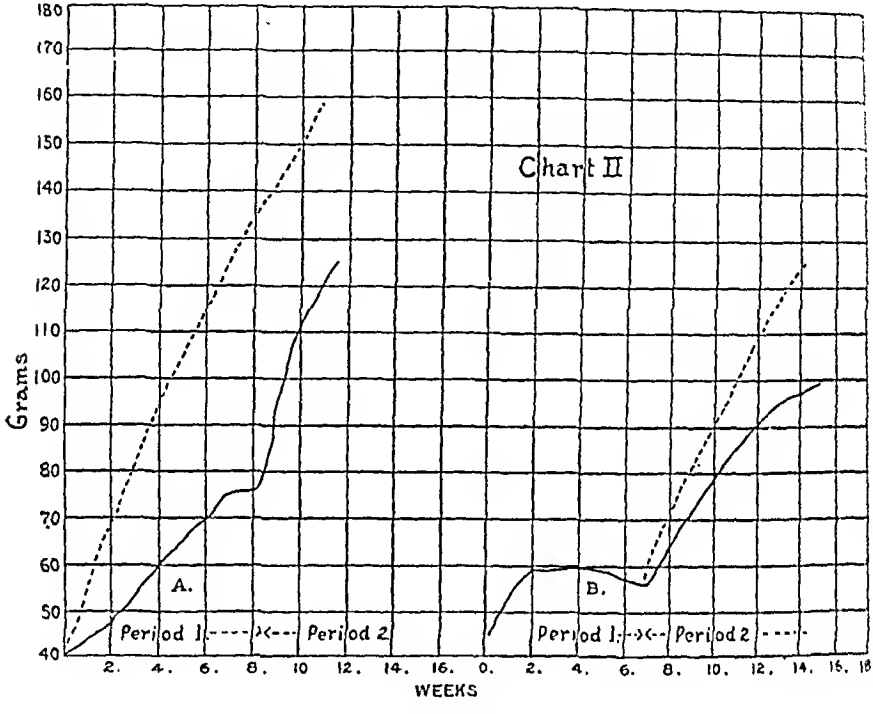


CHART II. Rat A (male). During period 1 this rat had the same fat-free ration given the rat in Chart I, but in addition received olive oil to the extent of 5 per cent of the ration. Period 2 shows the marked change in the rate of growth when olive oil from the same lot was shaken with a solution of the soaps prepared from butter-fat was substituted in the same amount as in period 1.

Dotted line shows the normal curve of growth.

Rat B (male). In period 1 the diet contained cotton seed oil, 3 per cent. In period 2 olive oil which had been shaken with the soaps prepared from butter-fat was substituted for the cotton seed oil. The curve of growth is still upward at the time of this writing.

Dotted line shows the normal curve of growth.

---

[5] Osborne and Mendel have also described work with such salt mixture. See *Proc. Soc. Exp. Biol. and Med.*, ix, p. 73, 1912; this *Journal*, xii, p. 81, 1912.

such diets. Normal growth during a period of somewhat more than one hundred days can be attained only by exceptional individuals. Many fail entirely to grow, others grow at decidedly under the normal rate. We believe that we have in such rations a means of measuring the vitality of individuals in a manner more satisfactory than any hitherto employed.

The individuals employed in these experiments were not of the most vigorous type. The influence of the olive oil treated as described above, with soaps prepared from butter fat, is none the less striking because of this fact. They indicate clearly that the substance or substances present in butter fat which exert such a marked stimulating action on growth are sufficiently stable to withstand the conditions of saponification we have employed. We have here a method which gives promise of leading to a separation of these constituents, if there be more than one, from butter fat and offers many possibilities for a further study of their nature. We have undertaken experiments in which the more readily volatile solvents are applied directly to the soaps without the use of oils as solvents for the biologically active substances. In this manner we hope to be able to isolate the constituents responsible for the resumption of growth when butter fat is added to the diet. We shall report on these experiments as soon as advisable.

Concerning the nature of the substances in butter fat responsible for the stimulating effect on growth nothing can be said at present. Osborne and Mendel[6] have affirmed that butter fat prepared by centrifugating melted butter, and pipetting off the clear fat, was "entirely free from nitrogen and phosphorus and was devoid of any ash-yielding, or water-soluble components." Funk and Macallum[7] have recently challenged this statement as regards nitrogen, since they find that butter fat prepared according to Osborne and Mendel's directions yields easily measurable quantities of nitrogen when washed with dilute acid. Five years ago one of us[8] called attention to the fact in connection with feeding experiments with purified food substances in which the sole object was to secure a ration free from all forms of phosphorus except the added inorganic phosphates, that clear butter fat obtained by

[6] Osborne and Mendel: this *Journal*, xvi, p. 424, 1913.
[7] Funk and Macallum: *Zeitschr. f. physiol. Chem.*, xcii, p. 13, 1914.
[8] McCollum: *Amer. Journ. of Physiol.*, xxv, p. 120, 1909.

melting butter and filtering the *clear fat* could be nearly freed from phosphorus only by thorough washing with dilute hydrochloric acid. We hesitate in questioning the statement of such distinguished investigators yet in the light of the experiences of Funk and Macallum and of our own, we feel that we must accept with reserve the statement that butter fat can be obtained free from nitrogen and phosphorus by so simple a procedure as centrifugating melted butter and pipetting off the clear fat.

The ration employed in the experiments reported in this paper had the following composition:

	Per cent
Casein	18.0
Dextrin	56.3
Lactose	20.0
Agar-Agar	2.0
Salt mixture	3.7

The salt mixture employed had the following composition:

	Grams
NaCl	0.173
$MgSO_4$ (Anhydrous)	0.266
$NaH_2(PO_4) + H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2H_2O$	0.540
Calcium lactate $Ca\ (C_3H_5O_3)_2 + 5\ H_2O$	1.300
Fe lactate (Merck)	0.118

THE DETERMINATION OF IODINE IN CONNECTION WITH STUDIES IN THYROID ACTIVITY.

By E. C. KENDALL.

(*From the Mayo Clinic, Rochester, Minn.*)

(Received for publication August 19, 1914.)

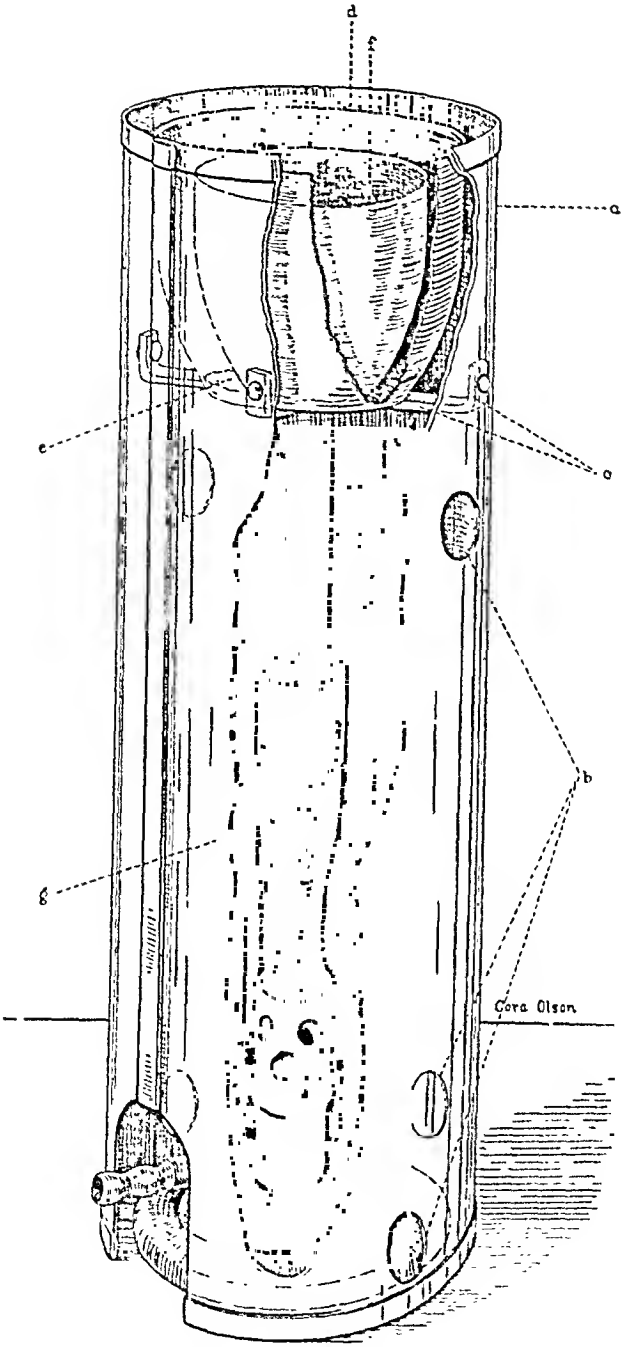
In July, 1912, the writer[1] published an article on the determination of iodine in the presence of chlorides, bromides and organic matter. Since that time the method has been in almost constant use for the estimation of iodine in connection with studies in thyroid activity. As several inquiries concerning the method have been received and as some of the details have been modified it seems desirable at the present time to describe the method with the modifications which have resulted from several hundred determinations of iodine.

The chemical reactions upon which the method is based are described in the original article and will not be given here. The determination of iodine when present in amounts ranging from 0.005 to 5 mgm. is as follows:

The first step is the destruction of organic matter and the retention of the iodine as sodium iodide. This is accomplished by fusion with sodium hydroxide in a nickel crucible. In order to maintain a proper degree of temperature and to prevent loss of iodine by volatilization, the crucible is heated indirectly by placing inside a larger crucible the bottom of which is covered with a layer of sand 0.5 cm. in thickness (see fig. 1). The drawing shows in detail the construction of the heating apparatus. The supporting cylinder is 9.4 cm. in diameter and 30 cm. high; the cross-bars which support the large crucible are 7.5 cm. from the top. Both crucibles are of pure nickel, the larger one 7.8 cm. and the smaller one 5.9 cm. in diameter. The burner is preferably a 15.6 cm. (No. 3) Meker burner.

For the determination of iodine in thyroid preparations, the

[1] Kendall: *Journ. Amer. Chem. Soc.*, xxxvi, p. 894, July, 1912.



a, Supporting cylinder of sheet iron; *b*, holes to supply air to burner; *c*, cross bars for supporting large crucible; *d*, large crucible 7.8 cm. in diameter; *e*, one-half cm. of sand on bottom of large crucible; *f*, fusion crucible 5.9 cm. in diameter; *g*, no. 3 Meker burner.

most satisfactory weight of the material to be analyzed is 0.5 gram. Whether this is in the form of a dry powder, a solution or a moist precipitate on a filter paper, the same procedure is carried out. The material is placed in a 5.9 cm. nickel crucible and moistened with 5 to 6 cc. of 30 per cent sodium hydroxide; 10 to 15 grams of stick sodium hydroxide which has been broken into small pieces are added and the crucible placed on a hot plate until the excess of water is evaporated and the contents have a thick, syrupy consistency. If but little organic matter is present, there is a tendency for spattering of fine drops during the evaporation of the excess water. If some organic substance is dissolved in the solution, this spattering is prevented. A small amount of gallic acid is suitable and sufficient for this purpose.

For the fusion of the organic matter with the sodium hydroxide, it is necessary to heat the bottom of the large crucible to a dull red heat. If the crucible is heated too much, the fusion in the small crucible will creep up the sides and sodium hydroxide will volatilize with loss of iodine. If the large crucible is not heated sufficiently, the destruction of organic matter will not be complete. However, there is a wide range of temperature between the two limits and after a little experience no difficulty is encountered.

When the sodium hydroxide is first heated in the presence of water, considerable foaming is produced. This, however, does not extend to more than half way up the sides of the crucible. As the heating continues, the foaming becomes less and after five to ten minutes the melt settles to the bottom. Bubbles will continue to be given off for some time (five minutes or so), depending upon the nature of the organic matter.

When the melt has settled to the bottom and only a few bubbles of gas are being liberated, the small crucible is removed with crucible tongs and partially cooled by agitating the contents with a rotary motion. This will also remelt and carry to the bottom any particles of the fusion which have solidified on the cooler sides of the crucible.

Five to ten milligrams of potassium nitrate are now added. This will oxidize the remaining organic matter and cause a liberation of bubbles. If only a few bubbles are liberated, a second addition of 5 to 10 mgm. of potassium nitrate will not cause a further liberation of bubbles and the oxidation of the organic

matter is complete. If the second addition of nitrate causes a further oxidation, repeated additions of 5 to 10 mgm. of the nitrate are made until no more bubbles of gas are produced by the addition of the nitrate. The melt is now poured into the shallow cover of the 5.9 cm. crucible and allowed to cool.

The entire time required for the fusion is ten to fifteen minutes. The writer finds it most convenient to use two crucible-supporting cylinders and to carry on two fusions at the same time.

When the melt and crucible are cool, they are placed in a tall beaker of from 600 to 800 cc. capacity together with a little talcum powder and 125 to 150 cc. of water. The beaker is placed on a hot plate. After the melt is dissolved, it is transferred to a 500 cc. Erlenmeyer flask. It should be a colorless, clear solution with a volume of about 200 cc. To the solution 1 cc. of 10 per cent of sodium bisulphite[2] and a few drops of methyl orange are added. The solution is cooled by immersing the flask in cold running water. When cool, 85 per cent phosphoric acid is added by allowing the acid to run directly into the flask from a pipette or syphon having a small delivery tube. The flask is vigorously and constantly shaken with a rotary motion to expel the carbon dioxide. As the indicator begins to turn pink the neutralization is finished slowly so that only a few drops are added after the indicator has changed. This is a very important condition to satisfy as too much acid will cause low results. A few drops of bromine are now added and the solution shaken until the bromine colors the solution a distinct yellow. This is essential as the addition of too little bromine will prevent the subsequent determination of the iodine.

The volume of the solution should be made between 250 and 300 cc. The flask is placed on the hot plate and boiled for eight to ten minutes. The talcum powder causes a rapid boiling and the bromine is quickly expelled. When the solution becomes colorless,[3] the flask is removed from the hot plate, five to ten drops

[2] The bisulphite is added to supply a reducing action to the solution which prevents the loss of iodine. As the iodine is kept in the form of hydriodic acid, no loss can occur. More than 1 cc. of bisulphite should not be added as it produces hydrobromic acid from the bromine and this in excess reduces iodic acid, giving low results.

[3] The bromine destroys the color of the methyl orange.

of 5 per cent solution of sodium salicylate[4] are added, and the flask is immersed in cold water. The volume of the solution after boiling should not be less than 175 to 200 cc. as the high concentration of the salts makes the end point less sharply defined if the solution is boiled down to a small volume.[5]

When the solution is cool, 5 cc. of 10 per cent potassium iodide are added. If there is not an immediate liberation of iodine, 3 to 4 cc. of 85 per cent phosphoric acid are added.[6]

The liberated iodine now titrated with $\frac{N}{200}$ sodium thiosulphate.[7] The titration is finished with aid of the blue starch-iodine color. For this, 0.5 per cent solution of Kahlbaum's soluble starch is recommended.

[4] This is best prepared by dissolving 5 grams of pure salicylic acid in dilute sodium hydroxide and diluting to 100 cc. The solution should be only slightly alkaline.

[5] Experience has shown that practically all samples of distilled water will take up small amounts of iodine. As this reducing action is destroyed by boiling with bromine, this source of error is entirely controlled under the conditions of the method as described above. However, a considerable error may result in the determination of iodine if any distilled water is added after the bromine has been boiled out of solution.

[6] The acid is added after boiling out the bromine rather than before, at the acidification of the fusion melt, as low results may occur when the solution is boiled in the presence of a large excess of phosphoric acid. No error is caused by adding even a large excess of the acid to the cold solution.

[7] The most convenient strength of sodium thiosulphate for amounts of iodine ranging from 0.5 to 5 mgm. is 0.005 N. This is not a stable solution and must be frequently restandardized. A convenient method proposed by Hunter is to prepare a solution of potassium acid iodate which is equivalent to a known weight of iodine. The strength of any sample of thiosulphate is readily found by titrating the iodine liberated by the acid iodate solution, which retains its strength indefinitely. The iodine equivalent of the potassium iodate is found as follows: Prepare an 0.1 N solution of potassium acid iodate KIO_3HIO_3 by dissolving 3.249 grams of the salt in 1 liter of water. This solution diluted twenty times will be approximately 0.005 N. Dissolve a known weight of pure iodine (approximately 1 gram) in 1 liter of water containing 5 to 6 grams of sodium hydroxide. Dilute this ten times. One cc. of this solution will contain 0.1 mgm. of iodine. Measure 25 cc. of this solution into a 500 cc. flask, and dilute to 200 cc.; add 5 to 10 drops 85 per cent phosphoric acid and a few drops of bromine; boil out the bromine; add 5 to 10 drops of 5 per cent sodium salicylate, cool, add 5 to 10 grams of sodium chloride, then add 5 cc. 10 per cent potassium iodine, and titrate the liberated iodine with approximately 0.005 N thiosulphate. This will establish the relation between "original iodine" and the 0.005 N thiosulphate,

In the opinion of the writer, the most convenient procedure where a number or determinations are made each day is to use six or seven crucibles each marked with a letter for identification. Each crucible has a corresponding 500 cc. Erlenmeyer flask marked with the same letter. The crucibles are used in rotation.

The methyl orange, bromine and sodium salicylate are kept in dropping bottles. The sodium bisulphite is kept in a 500 cc. bottle fitted with a graduated pipette which passes through the cork and reaches to bottom of the bottle. The 85 per cent phosphoric acid, the 10 per cent potassium iodide and the starch solution are kept in bottles fitted with syphons. This form of container prevents contamination and offers a convenient means of supply for the respective solutions.

In regard to the accuracy and limitations of the method, the results of over two thousand determinations of iodine show that the method will detect as little as 0.005 mgm. of iodine. One great advantage of the method is the entire absence of any test for iodine in cases where there is no iodine present.

Where iodine ranges from 1 to 5 mgm. duplicate determinations should not differ more than 0.01 to 0.02 mgm., which means a difference in burette readings of but 0.1 to 0.2 cubic centimeter.

As this investigation was begun and in large part completed in the Pathological Department of St. Luke's Hospital, New York City, I wish at this time to thank Dr. F. C. Wood for the opportunity of carrying on the work in that institution.

and from this the iodine equivalent of the potassium acid iodate can be found by adding a known volume of the acid iodate to 150 cc. of water containing potassium iodide, and 5 drops of 85 per cent phosphoric acid and 5 to 10 grams of sodium chloride. When a small amount of iodic acid is in a solution which contains but a small amount of salts the reaction with potassium iodide is retarded and the end point of the titration with thiosulphate is uncertain. The addition of 5 to 10 grams of sodium chloride to such a solution accelerates the liberation of iodine and makes the end point sharp and accurate. The sodium chloride must not be added until *after* the solution has been boiled with bromine.

THE FORMATION OF HEXONE AND PURINE BASES IN THE AUTOLYSIS OF GLOMERELLA.

By HOWARD S. REED.

(*From the Laboratories of Plant Pathology and Bacteriology, Virginia Agricultural Experiment Station, Blacksburg, Va. Paper No. 31.*)

(Received for publication, August 21, 1914.)

The fungus *Glomerella rufomaculans* is a parasite on the fruit of the cultivated apple and as such causes a decay involving profound changes in the composition of the fruit. Some of the enzymic activities of the fungus have been studied in this laboratory.[1] The present paper deals with certain questions concerning the cycle of nitrogen during the growth and autolysis of the fungus in pure culture on media of known composition. The studies of which this work forms a part are intended to throw light on the question of the nature of the injury of the parasite to its host.

Pure cultures of the fungus have been grown upon Czapek's solution which has the following composition:

Distilled water	1000 cc.
Magnesium sulphate	0.5 gram
Dipotassium phosphate	1.0 gram
Potassium chloride	0.5 gram
Ferrous sulphate	0.01 gram
Sodium nitrate	2.0 gram
Dextrose	30.0 gram

Large glass bottles having flat sides and known to the trade as "Short Blakes" were used for culture flasks. 150 cc. of solution was placed in a 1 liter bottle, the mouth plugged with cotton and sterilized fractionally in steam. After inoculation with the fungus the bottles were laid horizontally on the shelves of the incubator

[1] Reed and Stahl: this *Journal*, x, p. 109, 1911; Reed: *Annual Report*, Virginia Agric. Exp. Stat., 1911–12; Reed: *Eighth Internat. Cong. Applied Chem.*, xix, p. 265.

room. The solution had a surface of about 164 sq. cm. and a depth of 1.5 cm. The fungus grew well on this layer of solution and quickly formed a firm mat like those described in the former article. In the course of eight to fourteen days spores were produced in acervuli considerably different from the normal type and then the entire culture began to grow black. When cultivated on Czapek's solution in which sucrose is employed, this blackening is not developed to such an intensity as when grown upon the dextrose-containing formula. The melanotic products soon find their way into the culture solution and darken it also. Coincident with these changes there is an autolysis of the mycelial mats of the fungus which causes them to lose their firmness. The manner of this autolysis in another fungus has been described by Dox,[2] who showed that autolysis caused a loss in weight of the mycelium amounting to about 50 per cent in thirteen weeks. He reported that the autolysis was greater in proportion to the exhaustion of the carbohydrates in the medium.. The rate of autolysis was increased by replacing the nutrient solution with distilled water after the fungus had made its normal growth.

The organic bases of the fleshy fungi (Basidiomycetes) have been extensively studied, and it has been shown that the powerful poisons of many fungi are bodies belonging to this class. In a recent paper, Winterstein, Reuter, and Korolew[3] have presented the results of studies on the chemistry of three edible fungi (*Boletus edulis*, *Agaricus campestris*, and *Cantharellus cibarius*). They report the presence of such purine bases as guanine, adenine, xanthine, and hypoxanthine; also hexone bases including arginine, lysine, and so called histidine. The arginine fraction seemed to contain several bases as well as trimethylhistidine.

Various investigators have found purine bases in the aethalia of Myxomycetes and in the sporophores of certain Basidiomycetes.

Urea was found in the capillitium of the puff-ball, *Lycoperdon Bovista*, L. by Bamberger and Landsiedl.[4]

Reinke and Rodewald[5] reported the presence of certain bodies of the purine series in *Aethalium septicum* L. among which they distinguished guanine and xanthine.

---

[2] Dox and Maynard: this *Journal*, xii, p. 227, 1912; Dox: *ibid.*, xvi, p. 479, 1913–14.

[3] *Die landwirthsch. Versuchsstat.*, lxxix–lxxx, p. 541, 1913.

[4] *Monatsh. f. Chem.*, 1903, p. 63.

[5] *Untersuch. aus dem bot. Laborat. d. Univ. Göttingen*, 1881, p. 47.

The auto-digestion of yeast has been found to give rise to xanthine bases such as xanthine, hypoxanthine, and guanine.[6]

The nitrogen content of the cultures of Glomerella was first studied. By reference to the formula of the synthetic medium used, it will be seen that the nitrogen was furnished in the form of nitrate. A series of nitrogen determinations was made each week from the third to the tenth week of the life of the cultures.[7]

For each analysis two cultures in the "Blakes" were filtered through paper and well washed. The mycelium and solutions were analyzed separately. The solutions were divided into three equal portions, one for the determination of ammonia, one for nitrates, and one for organic nitrogen. The nitrates were determined as ammonia after reduction with zinc and iron, ammonia was distilled off with magnesium oxide, and the organic nitrogen determined by the Kjeldahl method. The results are given in Table I where the figures show the milligrams of nitrogen in different forms in the cultures. Since the cultures had individual variations the figures necessarily vary somewhat. The general trend of the results is to be taken therefore rather than individual analyses.

TABLE I

Distribution of Nitrogen in Glomerella Cultures

In Mycelium		In Solution		
Weeks	Total N	Organic N	Ammonia N	Nitrate N
0				40.00
3	29.30	9.90	0.30	0.65
4	31.60	6.70	0.68	0.50
5	19.50	15.60	3.08	trace
6	18.77	22.10	1.96	trace
7	20.60	13.71	3.50	
8	18.70	15.00	4.70	
9	18.92	12.88	1.96	
10	17.94	13.42	2.10	

These figures show that the nitrogen furnished as sodium nitrate is rapidly taken up by the growing fungus and a part then

[6] *Zeitschr. f. physiol. Chem.*, iii, p. 284, 1879.

[7] For these determinations I am indebted to Mr. Bruce Williams of this laboratory.

migrates back into the culture liquid. The amount in the mycelium remains quite constant after four or five weeks. The organic nitrogen in the solution which reaches a maximum sometime after autolysis begins, gradually falls off, due perhaps to the formation of ammonia or other easily volatile compounds. The amount of ammonia, which is the last to reach its maximum, in turn declines toward the tenth week.

It thus appears that ammonia is one of the compounds resulting from the decomposition of the organic nitrogen of the fungus.

Amines were sought for in the culture liquid, but no indication of their presence could be found.

The culture medium was next examined for the hexone bases, histidine, arginine, and lysine. These substances are open-chain amino-acids, which are well known dissociation products of proteins, but have not been isolated from any of the lower fungi until recently. Sullivan[8] reports the presence of hypoxanthine, guanine, adenine, and histidine in the mycelium of Penicillium, and in the culture solution on which it grew he found purine bases and a small quantity of a histidine-like body.

Cultures of *Glomerella rufomaculans* which had grown for four months on Czapek's solution were filtered and the filtrate examined for the three hexone bases above noted. The mycelium was converted into an enzyme powder using the acetone method described elsewhere.[9] The filtrate was analyzed for histidine, arginine and lysine according to the method of Kossel and Kutscher[10] and also used by Schreiner and Shorey.[11] This method of isolation depends on the fact that the two first named compounds are precipitated by silver salts from alkaline solution, while the third is precipitated by adding phosphotungstic acid to the acidified filtrate.

The analysis for histidine gave a small quantity of material from which the dichloride was obtained in its characteristic crystals. Unfortunately the quantity was too small to permit of making a melting point determination. Evidence of the presence of histidine was obtained however by the use of Pauley's diazo-

[8] Sullivan: *Science*, xxxviii, p. 678, 1913.

[9] Reed: *loc. cit.*

[10] *Zeitschr. f. physiol. Chem.*, xxxi, p. 166, 1900.

[11] This *Journal*, viii, p. 381, 1910.

benzene sulphanilic test on the culture medium. When treated with this reagent the solution became cherry red at once and the color remained after diluting with water. A small quantity of crystals resembling those of arginine nitrate were obtained but the quantity was too small to permit of positive identification.

Lysine which was found in this culture liquid is one of the most widely distributed cleavage products of proteins. Its presence was established by the method of isolation, by the characteristic crystals of lysine picrate, by the melting point of the dihydrochloride, and by the solubility of the picrate in water and relative insolubility in absolute alcohol.

Further information upon the nature of lysine production was acquired by using the enzyme powder prepared as previously mentioned from the fungous mycelium. A small flask containing 75 cc. of 7 per cent gelatin, received 0.5 gram of the enzyme powder and a few cubic centimeters of toluol. The flask was incubated for fourteen days. At the expiration of that time the solution was acidified with sulphuric acid, heated on the steam bath, and filtered. The filtrate was then treated with phosphotungstic acid and analyzed for lysine according to the method previously employed. A small amount of lysine picrate was obtained, showing that this substance may be produced from gelatin by the action of an enzyme which the fungus produces.

Another series of analyses was made for some of the purine bases. These bases are known to exist in plants, but are believed to arise through the disintegration of nucleoproteins. The methods of analysis employed were practically those described by Schreiner and Shorey.[12] Xanthine and hypoxanthine were found in the culture liquid. Xanthine was identified by the characteristic crystals of xanthine silver nitrate obtained by treating the ammoniacal silver nitrate precipitate with boiling dilute nitric acid and allowing the filtrate to concentrate slowly. A small quantity of the substance was evaporated on a watch glass with nitric acid leaving a yellow residue. When the residue was treated with sodium hydroxide and heated it became red and finally purple; thus corresponding to the "xanthine test."

Hypoxanthine also occurs in the culture liquid in considerable

[12] This *Journal*, viii, p. 385, 1910.

amount and was identified by (*a*) the method of isolation, (*b*) the crystalline appearance of the hypoxanthine silver nitrate, (*c*) and the red color developed when evaporated with nitric acid and bromine water and then treated with sodium hydroxide solution.

From these results it appears that the process of autolysis of Glomerella attacks the proteins of the fungous mycelium and produces certain hexone and purine bases in addition to ammonia.

STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE DOMESTIC FOWL.

IX. ON THE EFFECT OF CORPUS LUTEUM SUBSTANCE UPON OVULATION IN THE FOWL.

By RAYMOND PEARL and FRANK M. SURFACE.

(*From the Biological Laboratory of the Maine Agricultural Experiment Station, Paper No. 71.*)

(Received for publication, August 24, 1914.)

It has been shown by Loeb[1] that one function of the well developed corpus luteum in the mammalian reproductive cycle is to inhibit ovulation. This fact appears to be firmly established for mammals by his observations and experiments. Ruge's[2] observations seem also essentially to confirm this result. This suggestion was also made earlier by Prenant[3] and Sandes and Skrobansky.[4]

In connection with the series of studies in progress in this laboratory on the physiology of avian reproduction it seemed a matter of considerable interest to determine whether an extract of mammalian corpus luteum substance would exercise a similar inhibitory action on ovulation in a bird, where, of course, there is normally nothing connected with the ovary which is either morphologically or physiologically homologous to the corpus luteum of the mammalian ovary. The experiments here reported were undertaken for the purpose of throwing light upon this question.

[1] Loeb, L.: *Journ. of Morphol.*, xxii, pp. 37–70, 1911; *Zentralbl. f. Physiol.*, xxv, no. 9; *Virchow's Archiv*, ccvi, pp. 278–303, 1911; *Deutsch. med. Wochenschr.*, 1911, no. 1 and other papers.

[2] Ruge, H.: *Arch. f. Gynaek.*, c, p. 1, 1913.

[3] Prénant: De la valeur morphol. du corps jaune, son action physiol. et therapeut. possible, *Rev. gén. d. sci.*, 1898.

[4] Cf. Zuntz, L.: Weibliche Geschlechtsorgane, *Oppenheim's Handbuch der Biochemie*, iii, 1, p. 377.

MATERIAL AND METHODS.

In these experiments twenty adult healthy fowls, each approximately one year old, were used. All were laying actively and steadily at the time. They were carefully chosen for the experiments on the basis of their trap-nest records during the preceding weeks. The long experience of the writers in the study of fecundity in the fowl[5] made it clear that in order to get critical results in an experiment of this kind it would be necessary, first, to take hens during the spring months where the normal reproductive laying cycle is at its height, and second, to use only such birds as are showing at the time a smooth and high rate of fecundity for a considerable period before the actual beginning of the experiment. If these precautions are not observed in any such experiment it is obvious that it will be quite impossible to draw any reliable conclusions as to whether any observed lowering of the rate of fecundity is due to a normal periodic change in the rate of laying of the bird, which would have occurred in the absence of any experimental interference, or on the other hand is a direct effect of the agent applied to the bird. The fact that it was possible to choose the experimental birds from a large number (over 500) of laying birds on the Station plant, and that our previous studies had made us able to choose such birds as would give clear-cut results in one direction or the other, were essentials to the critical carrying out of the investigation.

The birds used were, for the most part, cross-bred individuals reared in connection with breeding experiments. In each case the hereditary nature of the bird will be indicated at the proper place. The treatment of the experimental birds following injection of corpus luteum, in respect of food, housing, care, etc., was exactly the same as their treatment before the experiment, and as that given the rest of the flock. At all times the experimental birds ran with the other birds on the plant, in flocks of about 125 birds each. It may be said here that in all of the experiments no harmful effects of any kind on the general physiological economy of the birds was observed to follow the injection save

[5] Cf. Pearl and Surface: A Biometrical Study of Egg Production in the Domestic Fowl, Parts I, II and III. U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 110, and other papers from this laboratory.

in the one case (cf. p. 270) in which death resulted from infection. The only observed physiological effect of the injection was the specific ovulation-inhibiting one presently to be described.

We have never observed any of the toxic effects which follow the intravenous injection of lutear extracts in mammals (rabbits, guinea-pigs and dogs), as described by Ferroni,[6] Lambert,[7] Villemin,[8] Burnam,[9] and others. Whether this apparent difference in the case of birds is due to differences in effective dosage, or to differences in methods of preparation of the extract, or substance, or to differences in tolerance between birds and mammals, or to a combination of all these factors we are unable to say.

It should further be said that no attempt was made to measure the blood-pressure in the birds following the injections. The investigators cited above, Hallion,[10] and others have noted a rapid fall in blood-pressure following intravenous injection of lutear extract. From the general behavior of our birds it seems very doubtful if there was any profound change in blood-pressure following the injection, in the present experiments.

The corpus luteum substance used in the experiments was the commercial dessicated gland substance put on the market by Armour and Company. This we understand to be simply the substance of fresh glands from cows. Fenger,[11] of the Armour laboratories, describes the "usual procedure" for collecting and preparing this substance in the following terms:

> The ovary containing the corpus luteum was removed from the animal shortly after slaughtering and while still containing the animal heat. The corpora lutea were then carefully dissected out and trimmed from all adherent tissue, weighed and stored at freezing temperature until the entire lot had been collected. The glands were finely minced, dried on agateware trays at a temperature not exceeding 65°C. (149°F.) to constant weight and extracted in the Soxhlet apparatus with petroleum benzin. The loss

---

[6] Ferroni, E.: *Ann di ostet. e ginec.*, i, 1907; *Folia gynaec.*, i, pp. 67–86, 1908.

[7] Lambert: *Compt. rend., soc. biol.*, lxii, p. 18, 1907.

[8] Villemin: *Le corps jaune considéré comme glande à secretion interne de l'ovaire*, Lyon (A Rey), 1908.

[9] Burnham, C. F.: *Journ. Amer. Med. Assoc.*, lix, pp. 698–703, 1912.

[10] Hallion: *Compt. rend. soc. biol.*, 1907, p. 40.

[11] Fenger: *Journ. Amer. Med. Assoc.*, lxii, pp. 1249–1250, 1914.

of moisture and petroleum-benzin-soluble material were noted, and the dissociated fat-free substance powdered in a tube-mill to pass a 60-mesh sieve.

The finished product is a dry fine powder. It still retains, in some degree, the characteristic yellow color of the fresh gland, which, as Escher[12] has lately shown, is due to an "animal" carotin, practically identical chemically with carotin from carrots. The dry corpus luteum is only partially soluble in 75 per cent NaCl and other watery solutions.

EXPERIMENTAL.

The general plan of the experiments was (1) to choose birds laying at a high and even rate, (2) inject either intra-abdominally or intravenously a definite amount of a suspension or an extract of the dessicated corpus luteum substance, and (3) observe the subsequent changes, if any, in the rate of fecundity. The results of the first two series of experiments will be exhibited in tabular form. In these experiments the amount of corpus luteum substance indicated in each case was mixed in an agate mortar with 11 cc. of sterile 0.9 per cent NaCl solution. This was injected cold, *i.e.*, without warming to body temperature of birds.

The first series of experiments were carried out with birds, which are relatively large and heavy specimens. The second series, and most of the subsequent experiments were performed on F_1 cross-bred birds from the cross Barred Plymouth Rock by Black Hamburg. These birds are of lower body weight than the pure Barred Plymouth Rocks.

[12] Escher: *Zeitschr. f. physiol. Chem.*, lxxxiii, pp. 198-211, 1913.

TABLE I.

Showing the effect of corpus luteum on barred Plymouth rocks. Intra-abdominal injections of corpus luteum suspension.

	BIRD NO.				
	K494 (control)	K472	K446	K497	K482
Variety	BPR	BPR	BPR	BPR	BPR
Body weight (gm.)	3040	3140	2280	2400	2720
Date (1914)	Mch. 24	Mch. 24	Mch. 24	Mch. 24	Mch. 24
Time of injection	11.30 a.m.	11.30 a.m.	11.30 a.m.	11.30 a.m.	11.30 a.m.
Amount of dry corpus luteum substance used (gm.)	0	0.1	0.5	1.0	1.5
Character of injection	Intra-abdominal	Intra-abdominal	Intra-abdominal	Intra-abdominal	Intra-abdominal
Eggs produced by bird in the 4 days preceding injection and the day of injection	3	5	4	4	4
Eggs produced in the 10th to 5th days preceding injection	4	2	3	3	4
Eggs produced in the 15th to 10th days preceding injection	4	4	0*	4	4
Eggs produced in the 1st to the 5th days following injection	4	3	2	1	0
Eggs produced in the 5th to the 10th days following injection	4	2	3	0	0
Eggs produced in the 10th to the 15th days following injection	4	4	3	2	0
Days after injection when first egg was laid	3	1	1	1 (12)†	16
Eggs laid in 10 days following first egg after injection	8	5	5	0 (5) †	10
Remarks	Bird had laid just before injection	Bird had laid just before injection	Bird had laid just before injection Died Apr. 28, 1914.	Bird had laid just before injection	Bird had laid just before injection Died Apr. 25, 1914.

* Bird had just begun to lay.

† In this and subsequent tables the figures in parenthesis have this significance that they represent the laying in the specified days, counting from the first egg *after* the egg laid on the day immediately following that of the injection, which egg was already in the upper end of the oviduct at the time of, or very shortly following, the injection.

From this table we note the following points:

1. The dosage of dry corpus luteum substance extended from 0.1 gram to 1.5 gram per bird. This corresponded to a dosage of from 0.03 gram to 0.55 per kilogram of body weight of the birds used.

2. In the control bird (K494) which received salt solution only, and in the two birds which got the two smaller doses of corpus luteum (K472 and K446) no appreciable effect on ovulation was produced by the injection. Such differences as appear in the egg laying before and after the injection are clearly of the same order as the fluctuations which may and do regularly occur in the laying of a normal bird.

3. In striking contrast are the results from the two birds K497 and K482, which received 1 gram and 1.5 gram of corpus luteum substance respectively. *In these birds ovulation immediately stopped after the injection* and was only resumed after the lapse of a considerable period of time (eleven days in the first and sixteen days in the second case).

4. The inhibiting effect of the corpus luteum substance on ovulation was apparently stronger in the case of the larger dose (1.5 gram) than with the smaller dose (1 gram), judged by the time which elapsed before the bird began to lay again. It should be understood that the egg laid by K497 on the day next following the injection was probably either already in the oviduct or just about to be separated from the ovary, at the time when the injection was made. It therefore cannot be taken as evidence of a more tardy action of the corpus luteum than in K482,

5. The recovery from the effect of the corpus luteum substance was apparently complete, as indicated by the subsequent laying, in those cases where there was a sufficient dose to inhibit ovulation. In other words the single injection did not permanently alter the ovulating mechanism.

6. Two of the birds used in this experiment died some time after the injection. In both cases the death occurred after a lapse of a little more than a month. In neither instance was anything found at the autopsy to indicate that the death was in any way connected with the fact that the bird had been used in this experiment. On the contrary there was every reason to suppose that these birds would have died at the time they did if they had never received any corpus luteum substance.

These experiments were repeated with another series of birds of different breeding, namely F_1 cross-bred birds from the cross Barred Plymouth Rock and Black Hamburg. These cross-bred birds were smaller than the pure Barred Rocks. The results are shown in Table II.

TABLE II.

Showing the effect of corpus luteum on cross-bred birds.

	BIRD NO.				
	K197 (control)	K220	K216	K128	K200
Variety	Hamburg Cross	Hamburg Cross	Hnmburg Cross	Hamburg Cross	Hamburg Cross
Body weight (*gm.*)	2030	2370	2060	2500	1610
Date (1914)	Mar. 24	Mar. 24	Mar. 24	Mar. 24	Mar. 24
Time of injection	11.10 n.m.	11.10 a.m.	11.10 a.m.	11.10 a.m.	11.10 n.m.
Amount of dry corpus luteum substance used (*gm.*)	0	0.1	0.5	1.0	1.5
Character of injection	Intra-abdominal	Intra-abdominal	Intra-abdominal	Intra-abdominal	Intra-abdominal
Eggs produced in the 4 days preceding and the day of injection	5	4	4	2	4
Eggs produced in the 10th to the 5th day preceding injection	5	4	4	4	4
Eggs produced in the 15th to the 10th days preceding injection	4	4	4	4	4
Eggs produced in the 1st to the 5th days following injection	3	2	1	1	1
Eggs produced in the 5th to the 10th days following injection	4	3	3	4	Bird died on fourth day following injection.
Eggs produced in the 10th to the 15th days following injection	4	4	5	2	
Days after injection when first egg was laid	1	3	1 (6)	1 (7)	
Eggs laid in 10 days following first egg after injection	7	5	4 (7)	4 (6)	
Remarks	In every case the bird had laid before injection in the morning of March 24.				

From this table the following points are to be noted:

1. On account of the smaller body weight of the birds in this series of experiments the dosage of corpus luteum substance

while absolutely the same as in the former series, is relatively larger, ranging from 0.04 gram to 0.93 gram per kilogram of body weight.

2. As before there is no effect of the injection in the case of the control (K197) and the 0.1 gram (K220) injections. The birds continue to ovulate practically as before, except for slight normal fluctuations in the rate. In the case of K220 it might be thought that a slight inhibiting effect had been produced, but a careful study of this bird's whole record convinces us that this was not in fact the case. The slight dropping off in the rate of fecundity following the injection would without doubt have occurred had there been no experimental treatment.

3. With the 1 gram dose just as before there is a distinct inhibition of ovulation following the injection, though the effect was here more transitory. This bird laid the day following the injection (egg in oviduct at time of injection). After that egg was deposited this bird did not lay again for seven days, and then resumed ovulation at a normal rate.

5. In the case of the highest dosage in this series of experiments, we unfortunately suffered the only loss of a bird in the whole investigation as a direct result of the operation. This bird died on the fourth day following injection. Autopsy showed it to be a case of general peritonitis, undoubtedly caused by infection at the time of injection.

On account of the death of K200 this experiment was exactly repeated on April 4, 1914, on another bird of the same breeding, and of body weight 2260 grams.

This bird was given 1.5 gram corpus luteum substance intra-abdominally in 11 cc. salt solution at 10.30 a.m. She had laid in the morning some time before the injection. She had been laying at the rate of 4 eggs in five days during more than thirty days preceding the injection. She laid the day following the injection (egg already in oviduct). She did not lay again for four days and in the ten days following the operation but 3 eggs were ovulated. She then resumed her normal rate of fecundity.

Taking all these cases of cross-bred birds together it appears that, as with the pure Barred Rocks, the injection of corpus luteum substance inhibits ovulation, if the dosage is sufficiently high. The effect did not however persist so long in the case of

these cross-bred birds as with the Barred Rocks in the preceding experiments. This probably means no more than that these were a somewhat more active lot of birds and eliminated the foreign substance more quickly. The cross-bred birds of this particular cross are in actual fact of a decidedly more active and nervous temperament than the pure Barred Rocks. Further support for the view suggested is found in the intravenous injection experiments to be described presently.

It has already been pointed out that the dessicated corpus luteum substance as received from the manufacturer is not very soluble in salt solution. It gives a muddy suspension when used in the proportions indicated in the preceding experiments. The thought occurs to one that the results obtained with the larger doses (1 gram and 1.5 gram) may not be due to any specific chemical action at all, but simply be the result of throwing suddenly into the peritoneal cavity some 11 cc. of a muddy suspension of proteins which has to be absorbed. This might conceivably so upset the bird as to inhibit ovulation for a time.

There are three different ways to test this question of whether the corpus luteum substance exerts a specific inhibiting action on the ovary or merely acts through mechanical disturbance of the equilibrium of the peritoneal absorbing mechanism. These are:

a. To inject intra-abdominally a filtered extract of the corpus luteum substance, which contains no solid particles (at least of more than microscopic size). If the action of the substance is due to a specific chemical such an extract should be equally effective, if sufficiently concentrated, in inhibiting ovulation. If on the contrary the action is due to a general mechanical disturbance such an extract should have no effect.

b. To inject intra-abdominally a large dose of corpus luteum substance in suspension after it has been inactivated by prolonged boiling. After this treatment the substance is mechanically essentially the same as before, but is chemically greatly changed. If the inhibiting action depends upon a specific chemical substance such boiled corpus luteum should be entirely inert and ineffective so far as specific ovarian activity is concerned.

c. To inject filtered extract of corpus luteum substance intravenously. This can cause no disturbance of the peritoneal

system, yet should be effective in inhibiting ovulation if this result depends upon a specific chemical substance.

All of these three classes of experiments have been tried with results which consistently indicate that the inhibiting action already described is due to a specific chemical substance which acts upon the ovary.

These results will now be described in detail.

Experiments with raw extracts injected intra-abdominally.

The results of these experiments are shown in Table III. The extracts of the dry corpus luteum substance were prepared in the following ways. In the case of bird No. 398, the first on which an extract was used, 6 grams of the dry substance was extracted at room temperature in 33 cc. of 0.9 per cent NaCl solution. The mixture was then filtered and centrifuged. As has been said the substance dissolves only partially and with difficulty.

Later a series of extracts were prepared in the following way, and used in all the extract experiments as indicated, except in the case of K398, as already mentioned. On April 9, 1914, three bottles were prepared each containing 5 grams corpus luteum powder to which 25 cc. of 0.9 per cent of sterile NaCl solution were added. These were thoroughly shaken. One bottle was allowed to stand at room temperature with frequent shaking. Another was heated in a water bath at between 50 and 60°C. for five hours. The third bottle was boiled for four hours in a water bath. They then stood at room temperature until the next morning, about twenty-four hours in all. On April 10, 1914, the extracts were poured into test tubes. The boiled extract had coagulated and evaporated until it was necessary to add 8 cc. of NaCl solution to wash out the bottle. Five cc. NaCl were added to the heated extract and 3 cc. to the raw extract. As shown by the height on the test tube all these extracts now had about the same volume. These were centrifuged and the fluid pipetted off. This was again centrifuged. The raw and treated extracts were still cloudy and these were run through filter paper, although this removed but little sediment and these extracts were still cloudy. The boiled extract was much clearer in color as if some of the substances had been thrown down by the boiling. Approximately 20 cc. of fluid were recovered from each sample. These were injected

as indicated in the tables giving the results of the extract injections made April 10 (Table III–IV).

TABLE 3.

Showing the results from injecting raw corpus luteum extract intra-abdominally.

	BIRD NOS.	
	K398	K195
Variety	Cross-bred	Cross-bred
Body weight (*gm.*)	2250	1860
Date (1914)	April 4	April 10
Time of injection	10 a.m.	11 a.m.
Amount of extract injected (cc.)	11	11
Character of injection	Intra-abdominal	Intra-abdominal
Eggs produced in the 4 days preceding and the day of injection	4	4
Eggs produced in the 10th to the 5th days preceding injection	4	3
Eggs produced in the 15th to the 10th days preceding injection	3	5
Eggs produced in the first to the 5th days following injection	0	0
Eggs produced in the 5th to the 10th days following injection	0*	2
Eggs produced in the 10th to the 15th days following injection	3	3
Days after injection when first egg was laid	12	9
Eggs laid in 10 days following first egg after injection	7	7
Remarks	Bird laid April 4 before injection	Bird laid April 10, before injection.

* There was an undoubtedly erroneous record made by the poultryman of one egg on Apr. 9 (i.e., 5 days after the injection). The bird did not actually begin laying until April 16. We have not taken account of this record on the 9th in the table, because from other evidence we feel certain that it was an error in the trap-nest operation (in this connection cf. Pearl: Me. Agr. Exp. Stat., Ann. Rept. for 1911, pp. 185–193, where the unavoidable errors of trap-nesting are discussed). Even if the record were allowed to stand the inhibition of ovulation following the injection is still perfectly clear.

From this table it is clear that the raw extract of the corpus luteum substance in normal saline produces exactly the same sort of inhibition of ovulation as when the substance itself is injected intra-abdominally. In one of the two birds the effect persisted twelve days and in the other nine days. Then ovulation was again resumed at the normal rate. The fact, shown by birds K494 and K197 in Tables I and II respectively, that when the same amount of 0.9 per cent NaCl solution is injected intra-abdominally no effect whatever is produced on ovulation proves that it cannot have been the mechanical result of injecting 11 cc. of fluid which stopped ovulation so immediately. The evidence is very strong that the extract must have contained some chemical substance to which this specific action is due.

The effect of injecting intra-abdominally a suspension of boiled corpus luteum substance.

A check experiment on this line was performed April 18, 1914. 1.5 gram of the dry corpus luteum substance was mixed up in 10 cc. of NaCl solution and boiled hard for 1–3.4 hours. At the end of this time enough salt solution (about 3 cc.) was added to bring up the total volume to 11 cc. The resulting suspension was in its physical consistency and make-up much like the suspension of raw substance injected in the original experiments set forth in Tables I and II. The only difference was that the boiled suspension was more pasty than the raw.

This boiled suspension was injected intra-abdominally at 11.10 a.m., April 18, 1914, into bird No. K158 (body weight, 1880 gm.) a BPR × BH F_1 cross-bred bird. The bird had laid on the morning of the 18th before the injection. *No inhibition of ovulation followed.* This bird laid on the day following the injection, skipped a day, then laid on the 21st and 22d, skipped the 23d, laid on the 24th and 25th, skipped the 26th and 27th, then laid 5 eggs in succession, skipped May 3d, then laid 10 eggs in succession and has continued to lay at perfectly normal and high rate. This bird's record for the fifteen days prior to and including the day of injection was, taken in 5-day periods, 5, 4 and 5. There was a slight slowing of the rate of laying in the first ten days following the injection, but clearly nothing approaching inhibition of ovu-

lation. The observed slowing in the rate would almost certainly have occurred had there been no injection. That is, it undoubtedly represents a normal fluctuation in rate of fecundity.[13]

This experiment, taken in connection with what has preceded, proves,

1. That the inhibiting action upon ovulation observed when raw corpus luteum suspensions are injected into the abdominal cavity cannot be due to any mere mechanical disturbance of the peritoneal absorbing mechanism which then interferes with the normal activity of the ovary, and

2. That the specific ovulation inhibiting substance is inactivated by boiling.

The latter conclusion is further demonstrated by injecting boiled lutear extract (prepared as described above, p. 272) intra-abdominally. On April 10, 1914, 11.45 a.m., there were injected intra-abdominally 11 cc. of such boiled, filtered extract into bird No. K184 (body weight 1930 gm.) The bird had laid on April 10 before injection. She did not lay on the 11th or the 12th, laid on the 13th, skipped the 14th and 15th, laid every day from and including the 16th to the 21st, and so on at a normal rate. Before the injection her rate in 5-day periods had been 4, 5 and 4. Clearly the boiled extract produced no inhibition of ovulation.

The effect of intra-venous injection of raw and boiled lutear extract.

In the experiments so far discussed the administration of lutear material has been intra-abdominal. Will the extracts give the same results when given intravenously? The results of experiments on this point are given in Table IV. The extracts used are those described on p. 272 as prepared for the injection of April 10.

From this table it is seen that neither the injection of salt solution (K232) nor the injection of boiled extract (K230) produced any effect whatever on ovulation.

The result with the raw extract is very different and interesting in several particulars. In the first place, while the injection did

[13] These normal fluctuations in fecundity rate will be discussed in detail in a paper shortly to be published by R. Pearl.

TABLE 4.

Showing the results of intravenous injection of lutear extracts.

	BIRD NO.		
	K232 (control)	K230	K203
Variety	Cross-bred	Cross-bred	Cross-bred
Body weight (*gm.*)	1460	1320	1570
Date (1914)	April 10	April 10	April 10
Time of injection	11 a.m.	11.40 a.m.	11.20 a.m.
Amount of extract injected	4 cc. of 0.9 % NaCl only.	4 cc. + 3 cc.	5 cc.
Character of injection	Intravenous.	4 cc. intravenous. 3 cc. subcutaneous.*	Intravenous.
Treatment of extract used	No extract used. Only pure NaCl.	Boiled	Raw
Eggs produced in the 4 days preceding and the day of injection	4	3	4
Eggs produced in the 10th to the 5th day preceding injection	3	3	3
Eggs produced in the 15th to the 10th days preceding injection	4	2	4
Eggs produced in the 1st to the 5th days following injection	3	2	3
Eggs produced in the 5th to the 10th days following injection	5	3	1
Eggs produced in the 10th to the 15th days following injection	3	3	0
Days after injection when first egg was laid	3	2	1
Eggs laid in 10 days following first egg after injection	9	5	4
Remarks	Birds laid April 10 before injection.		

*This injection went badly. 1 cc. was first put in one brachial vein and then, owing to cutting of the vein, the other wing was taken and 3 cc. more put into the brachial vein there. Then to make sure of sufficient dosage 3 cc. were given subcutaneously.

not *immediately* inhibit ovulation as did the intra-abdominal injections, it did so eventually in a very marked degree. This is shown by the complete record of production following the injection.

April 10 (injection)	laid 1 egg.
April 10	laid 1 egg.
April 12–13	laid 0 egg.
April 14	laid 1 egg.
April 15	laid 1 egg.
April 16	laid 0 egg.
April 17	laid 0 egg.
April 18 to April 30, inclusive	laid 0 egg.
May 1	laid 1 egg.

After the inhibition of ovulation became complete on May 18 the bird did not lay again for thirteen days. So there can be no doubt of the fact of inhibition here, but it is not altogether clear why it is delayed, as compared with the intra-abdominal. It may be due to a very rapid elimination of the bulk of the substance when given intravenously, which, if the case, would indicate the need for a different dosage to get the same result as has been described in the intra-abdominal work. It is idle to speculate as to the cause of the difference until many more experimental results are at hand. The important thing, from the standpoint of this first study, is that lutear extract when given to the bird intravenously does inhibit ovulation.

Some preliminary experiments were tried to find the temperature limits of thermostability of the active substance which inhibits ovulation, but these will not be reported at the present time, since they were only preliminary, and since further we hope to continue our study of this matter next year, at the season when the hens are laying at a high and regular enough rate to make critical work possible. We intend then to proceed as far as possible in the chemical isolation of the active ovulation inhibiting substance of the corpus luteum.

DISCUSSION.

We shall make no attempt to discuss in detail the general literature on the corpus luteum as an organ of internal secretion beyond the references which have been made in the body of the

paper. Such discussion may more profitably be undertaken after further work has been done in the direction of chemically isolating the ovulation inhibiting substance.

The fact demonstrated in this paper that mammalian corpora lutea contain a chemical substance which inhibits ovulation in a bird is one of interest zoölogically as well as physiologically. It adds another to the list of vital processes known to be directly and specifically controlled chemically. It suggests that, since it is possible to obtain a chemical substance which *inhibits* ovulation, without in any way otherwise affecting the normal vital processes of the bird, it will also probably be possible to find a chemical substance which has the opposite action, and will stimulate, or better activate, the ovulation mechanism. We hope shortly to undertake experiments in this direction.

The fact that the same chemical substance inhibits ovulation in mammals and birds, which latter do not possess any organ corresponding to the one which produces the substance in mammals (the corpus luteum) suggests that natural selection probably had nothing to do with the evolution of either the organ or the function in the mammals. If it had, one would expect that the function would have been perfected as a specifically and exclusively mammalian one. What we find in respect to this ovulation inhibiting function of the corpus luteum is that it is specific *physiologically* (or chemically) but not specific *taxonomically*. Yet the latter is what would be expected had the function been developed and perfected through the action of natural selection.

SUMMARY.

In this paper it is shown that the dessicated fat-free substance of the corpus luteum of the cow, when injected in suspension, in proper dosage, into an actively laying fowl immediately inhibits ovulation. The duration of this effect varies with different birds from a few days up to two to three weeks. After the bird begins ovulating again the laying goes on unimpaired. The same effect is produced by the injection of extracts of the lutear substance, either intravenously or intraabdominally. The active substance in producing the inhibition is inactivated by boiling.

Further investigation of the subject is in progress.

THE PURINE ENZYMES OF THE OPOSSUM (*DIDELPHIS VIRGINIANA*).

By GEORGE T. CALDWELL and H. GIDEON WELLS.

(*From the Pathological Laboratory of the University of Chicago and the Otho S. A. Sprague Memorial Institute.*)

(Received for publication, August 27, 1914.)

In view of the peculiarities of the distribution of the purine enzymes in the tissues of different species of animals, we have taken advantage of an opportunity to investigate the purine enzymes of a marsupial, the common American opossum. The material was kindly placed at our disposal by Dr. R. R. Bensley, to whom our indebtedness is hereby expressed. The several enzymes were sought for by the usual methods, as described in previous papers,[1] and the protocols are given below.

SERIES 1. DIGESTION OF URIC ACID AND XANTHINE WITH AIR CURRENT.

Experiment 1. Liver tissue, ground fine; 19.5 grams allowed to act forty-eight hours at about 37° on 0.176 gram uric acid and 0.176 gram xanthine, with a current of air drawn through the mixture. After this digestion there could be recovered no uric acid and a mere trace of xanthine; indicating the presence of uricase and xanthine-oxidase.

Experiment 2. Fifty grams liver tissue acted on 0.101 gram xanthine with air current forty-eight hours in as Experiment 1. No uric acid or xanthine could be recovered, showing the presence of uricase and xanthine-oxidase in the opossum liver, and confirming the result of Experiment 1.

Experiment 3. Fifty grams liver tissue acted on 0.191 gram

[1] This *Journal*, vi, pp. 321 and 469, 1909; vii, p. 171, 1910; xv, p. 449, 1913; xviii, p. 157, 1914.

uric acid, with air current as above. From this digestion we could recover no uric acid or xanthine, again showing the presence of uricase, and presumably also of xanthine-oxidase.

Experiment 4. Twenty-five grams fresh kidney tissue from two opossums emulsionized, and allowed to act forty hours at 36-42° with air current on 0.152 gram xanthine. Recovered 0.024 gram uric acid, 0.104 gram xanthine, and small amounts of what may have been guanine and hypoxanthine. The uric acid was identified by its crystalline structure and the murexide test. Evidently the kidney has some power to convert xanthine into uric acid, but this is very slight as compared with other active tissues. The quantity of uric acid plus xanthine recovered is so near that added originally, that the presence of uricase is excluded.

Experiment 5. Fifteen grams of emulsionized kidney tissue acted on 0.150 gram xanthine with an air current, as in Experiment 4. From this recovered no uric acid, and 0.094 gram xanthine. The presence of so much xanthine after this length of time and the absence of uric acid, indicates the absence of active xanthine-oxidase from the kidney of this opossum, although this is in contradiction to the result obtained in Experiments 4 and 11.

Experiment 6. A mixture of spleen, lungs, adrenals, testicles and intestines, amounting in all to 14 grams, acted upon 0.188 gram uric acid and 0.178 gram xanthine, under the same conditions as in the above experiments. Here there were recovered 0.192 gram uric acid and 0.104 gram xanthine, no adenine, and possibly traces of guanine and hypoxanthine. In these tissues no uricase or xanthine-oxidase seem to be present.

SERIES 2. DIGESTION OF ADENINE AND GUANINE WITHOUT AIR.

Experiment 7. Liver, 85 grams, acted at 37° for two weeks under toluene in a tightly stoppered bottle on 1.052 gram adenine hydrochloride (equals 0.114 gram adenine) and 0.153 gram guanine-hydrochloride (equals 0.117 gram guanine). From this mixture we recovered 0.218 gram xanthine, 0.030 gram adenine, and 0.085 gram hypoxanthine; no uric acid or guanine recovered. Evidently the liver contains guanase. The low recovery of adenine and large amount of hypoxanthine suggests the presence of adenase, but does not prove it, since quantitative recovery of adenine from these mixtures is difficult. However, the same low

recovery of adenine in the following experiment, and the result of the control, support the evidence of a weak adenase action in opossum liver.

Experiment 8. Thirty-six grams of liver tissue from two young opossums was digested at 37° under toluene for twelve days, with 0.153 gram adenine hydrochloride (0.114 gram adenine) and 0.177 gram guanine hydrochloride (0.135 gram guanine). From this there was recovered 0.056 gram adenine, 0.064 gram hypoxanthine and 0.160 gram xanthine; there is no guanine or uric acid present.

A duplicate experiment was performed in which the liver tissue was boiled before adding the purines and incubating. Here we recovered 0.115 gram guanine, no xanthine, 0.096 gram adenine, and 0.038 gram of hypoxanthine, proving the presence in the unboiled liver of active guanase and speaking in favor of the presence of a slight adenase activity.

Experiment 9. Muscle, 20 grams, tested as above with 0.114 gram adenine and 0.117 gram guanine. Recovered 0.124 gram xanthine, 0.081 adenine, 0.031 hypoxanthine, and no uric acid or guanine. Here guanase alone seems to be present.

Experiment 10. A mixture of viscera (spleen, lungs, adrenals, testicles and intestines) weighing 48 grams, digested with 0.114 gram adenine and 0.117 gram guanine. Recovered 0.137 gram xanthine, 0.064 gram adenine and 0.038 gram hypoxanthine. No uric acid or guanine found. Here the only enzyme present seems to be guanase.

Experiment 11. Twenty grams of kidney tissue, conditions as in Experiments 9 and 10. Recovered 0.045 gram of typical uric acid crystals, giving a positive murexide test; 0.101 gram xanthine, 0.081 gram adenine and 0.031 gram hypoxanthine; but no guanine. Here guanase is certainly present, and the presence of xanthine-oxidase is suggested by the finding of uric acid; adenase seems to be absent.

In a large number of similar experiments performed in this laboratory with tissues of many sorts, this is the first and only time that uric acid has been produced during anaerobic digestion. The repetition of this experiment, given below, failed to give the same result. It is interesting to note that the aerobic digestion of xanthine by kidney tissue (Experiments 4 and 5) also gave

contradictory results, there being here also with one experiment a slight formation of uric acid, and none with the other.

Experiment 12. Kidney tissue, 14 grams, acted on 0.114 gram adenine and 0.135 gram guanine anaerobically for twelve days. Recovered 0.074 gram adenine, 0.025 gram hypoxanthine, and 0.092 gram xanthine, but no uric acid or guanine. This experiment corroborates the conclusion as to the presence of guanase and the absence of adenase, but does not demonstrate the presence of xanthine-oxidase.

To recapitulate, our results indicate that the liver of the opossum contains uricase and xanthine-oxidase, as shown by the destruction of these two purines by emulsions of liver tissues in the presence of an abundant supply of oxygen. Uricolytic enzymes could not be found in any other tissues of the opossum. With one of two specimens of kidney which acted on adenine and guanine without air being present, there was recovered a considerable amount of uric acid; and of two repetitions of the experiment with kidney tissue and xanthine acting in the presence of air, one failed to yield any uric acid whatever while the other yielded an appreciable quantity of uric acid. Apparently, then, the kidney tissues possesses to a very slight degree the power of oxidizing xanthine to uric acid, and this presumably depends on the presence of xanthine-oxidase in this organ.

All tissues examined converted added guanine into xanthine quantitatively, *i.e.*, guanase is widely distributed. We could not positively demonstrate the presence of adenase in any tissue. To be sure, we failed to recover added adenine quantitatively from our digestion mixtures, but we do not look upon anything less than total or almost total disappearance of adenine as proof of the presence of adenase in tissues, in view of the difficulty in recovering adenine quantitatively from digestion mixtures. The loss of the adenine with liver tissue, however, was regularly higher than could be satisfactorily explained as analytic error, especially when contrasted with a boiled control specimen, so that it seems probable that the opossum liver does possess a slight adenase activity.

The only other recorded observations on the purine metabolism of the opossum is given by Hunter and Givens,[3] who found that

[3] This *Journal*, xviii, p. 398, 1914.

this animal excretes relatively more of its purine nitrogen as uric acid than most other mammals, the "uricolytic index" varying from 73 to 89. These figures do indicate, however, the presence of active uricolytic enzymes in the opossum, which inference is established by our direct observations.

SUMMARY.

In the tissues of the opossum (*Didelphis virginiana*) enzymes acting on free purines *in vitro*, were found distributed as follows: Uricase, present only in the liver; xanthine-oxidase, present in the liver, and probably in the kidney; guanase, present in all tissues; adenase, probably not present in any of the tissues, except possibly in the liver.

ON THE DETERMINATION OF SUGAR IN BLOOD.

PHILIP A. SHAFFER.

(From the Laboratory of Biological Chemistry, Washington University, St. Louis.)

(Received for publication, August, 28, 1914.)

It is becoming more and more apparent that a knowledge of the chemical composition of the intake and of excreta is inadequate for the solution of many problems concerning the transformations of material in the animal body, and that it is necessary to look in locations closer to the cells for evidence as to the mode of metabolism in both normal and pathological conditions. Such considerations have doubtless in part given rise in the past few years to the widespread and increasing interest in the technique for the chemical analysis of blood and tissues.

The very important work of Folin and Denis, and of Van Slyke and Meyer on the absorption and intermediary catabolism of protein and its derivatives, and the work of Michaelis, Rona, Frank and others on blood sugar, already indicate how much may be expected from the application of an accurate scheme of blood analysis to the problems of metabolism. Other possibilities in this direction are shown by the work in this laboratory of Bloor on blood-fat and of Marriott on "acetone bodies" in blood, and by the work of Myers and Fine, Folin and Denis, and the writer on creatine and creatinine.

It is the purpose of the present paper to describe several modifications of technique for the determination of blood sugar which we have used during the past year and which it is believed have important advantages over other methods in ease and convenience of operation and, for small amounts of blood, a greater accuracy.

It early became evident to us that to be of use under ordinary experimental conditions, a method for the determination of blood sugar must require only 5 or 10 cc. of blood, because the withdrawal of larger amounts at one time often causes a disturbance of the sugar concentration. The method finally adopted permits

the determination in 5 cc., an amount which may be readily obtained even from rather small animals, or may, if desired, be repeatedly drawn (from dogs at least) during the same day without so far as we have observed, affecting the sugar concentration. The method calls for but few manipulations and, once the apparatus and solutions are assembled, may be carried out within an hour.

The principal modifications of the technique consist in the removal of the proteins by combined heat coagulation and the Michaelis-Rona colloidal iron precipitation,[1] and the use at several points of the centrifuge and centrifuge tubes. In this way the whole process is greatly shortened and simplified; the blood extract need not be greatly diluted, as is usually done, and the filtrate may be used for the reduction of copper solutions without previous evaporation; the reduction takes place in centrifuge tubes, after which there is no transfer of the cuprous oxide and but little opportunity for its reoxidation.

Drawing of blood. The importance of this part of the technique in a quantitative study of blood sugar is, I believe, still not sufficiently realized, in spite of the fact that the points involved have been repeatedly discussed at length. There is no doubt that in all experimental work the conditions under which the blood is drawn are of even greater importance than the accuracy of the analytical technique for the determination of the sugar (see the following paper). Further discussion of the matter here is unnecessary except to say that if one desires to know the concentration of blood sugar in the naturally circulating blood of an intact animal or human subject, the blood must be drawn without in any way causing fright, pain, excitement or struggling. It is therefore *not* permissible to give a general anaesthetic, to make an incision or lay bare the vein or artery, unless of course it be one of the above factors, the effect of which is being studied.

With a few exceptions we have worked only with dogs and with human subjects. With dogs we have always drawn the blood

[1] The use of heat coagulation together with colloidal iron precipitation has already been advocated by Michaelis (*Biochem. Zeitschf.*, lix, p. 166, 1913). I had been using the procedure described for some time before I knew of Michaelis' paper. The procedure is convenient and effective and should prove useful also for other purposes.

from the jugular vein or from the femoral artery. The skin is shaved and cleaned (in strange or excitable animals, some hours before the blood is to be drawn), and the animal is gently tied, back down, to a comfortable board, or is held firmly and quietly in the lap of an assistant who in either case holds the head extended. The animal must be quiet and free from excitement. Dogs will usually lie quiet in almost any position if the abdomen or chest walls are stroked or scratched gently.

The jugular vein is then distended by pressing a finger over the base of the neck on one side, and a *sharp* medium size needle, attached to a short piece of rubber tubing, is plunged through the skin and into the vein just beneath. With a little practice there is usually not the slightest difficulty in drawing in this way as much blood as needed without causing any excitement of the animal. The blood may be merely allowed to drop from the rubber tubing into a flask containing a little powdered potassium oxalate from which it is later weighed or measured, or more conveniently, as described by Folin and Denis, the blood is collected and measured at once in the pipettes containing a few grains of powdered potassium oxalate, the tip of the pipette being inserted into the rubber tubing attached to the needle. A little suction applied to the pipette hastens the flow and decreases the danger of clotting. We draw in this way 5 cc. of blood which is run at once into a 100 or 200 cc. Erlenmeyer flask containing 25 cc. of water; the pipette is rinsed out twice with the same water.[2] With human subjects the blood is drawn from a vein of the arm, as is usually done.

The removal of proteins. For the removal of proteins the flask, covered with a small watch glass, and held at the neck with a test-tube holder, is heated quickly over a small flame *just to boiling.* During the heating the flask should be rotated gently; evaporation is to be avoided. A few drops of dilute acetic acid are at once added to the hot liquid to produce a visible coagulum. Five cc. of colloidal iron solution (iron dialyzed, Merck) is next added and

[2] If duplicate determinations are desired, 10 cc. of blood are drawn and the amounts of water and the other reagents mentioned above are doubled. If the sugar in the plasma rather than in whole blood is desired, 10 cc. of blood is drawn into a small centrifuge tube, mixed with a little powdered oxalate and centrifugated *at once* for ten minutes. Five cc. of the plasma is pipetted out and treated as above described for whole blood.

the mixture well shaken; after which about 0.2 gram of powdered sodium sulphate is added and the mixture again shaken for about ten seconds. The liquid is then poured as completely as possible into a 50 cc. centrifuge tube and centrifugated at moderate speed for one or two minutes.[3] The water-clear liquid is then poured off through a small filter and 21 cc., equivalent to 3 cc.[4] of blood, are measured into another 50 cc. glass centrifuge tube. Twenty-one cc. of mixed Fehling's-Allihn solution[5] is then added and the tube immersed in boiling water, to the height of the liquid inside the tube, for ten minutes.

The centrifuge tubes are of rather heavy glass, but are fairly resistant to even such sudden changes of temperature. It is, however, important

---

[3] We use the No. 1 centrifuge of the International Instrument Co.

[4] The total volume is 36 cc. (or 72 cc. for 10 cc. of blood); 1 (or 2) cc. being allowed for the volume of the precipitate, 7 cc. of the filtrate represents 1 cc. of blood.

[5] The solutions we have used are the following:

I. 69.28 grams crystalline copper sulphate in 1000 cc.
II. 346 grams sodium potassium tartrate and 250 grams potassium hydroxide in 1000 cc.

For the determination of only a few milligrams of glucose by copper reduction methods the conditions as regards concentration of alkali and of copper, the manner and time of heating and the opportunity for reoxidation of the copper are especially important. The optimum concentration of alkali and copper appears to be obtained by adding an equal amount of freshly mixed Fehling's-Allihn solution to the solution containing the sugar, when the final concentrations are approximately the following: 6.2 per cent KOH, 9 per cent tartrate, and 1.8 per cent $CuSO_4.5H_2O$. With lower concentrations of either alkali or copper, the amount of cuprous oxide formed by small amounts of glucose (1 mgm. in 20 cc.) is less. According to the method recently described by Michaelis (*Biochem. Zeitschr.*, lix, p. 166, 1913), the reduction is carried out in a total volume of about 15 cc. (from 0.6 cc. of blood), containing about 0.4 mgm. glucose and approximately 0.75 per cent NaOH and 0.15 per cent $CuSO_4.5H_2O$, or about one-tenth the concentrations which I have used. With such concentrations I find the reduction small and variable.

The Michaelis method is similar in many ways to that here described, except that he uses 1 cc. or less of blood. As a result of my experience I doubt the wisdom of attempting a determination by copper reduction methods in amounts of blood containing less than 2 mgm. of glucose. Accordingly, no less than 4 or 5 cc. of normal blood can well be used, allowing about half for loss in the protein precipitation. The results are somewhat more accurate with still larger amounts.

that they should not be in contact with metallic supports when plunged into the hot water. I have found very satisfactory and convenient for this purpose circular racks, made similar to test tube racks, but constructed of "fibre board" which is very resistant to the hot water. A rack about 15 cm. in diameter has space for six tubes and of course permits the heating of all at one time.

On removal from the water bath the tubes are placed in the centrifuge (each pair being balanced by the addition of water if necessary) and centrifugated for two to four minutes, which packs the cuprous oxide in the bottom of the tube. The liquid is then carefully poured off as completely as possible without loss of oxide. This should be done over a white evaporating dish and in a light place which permits one to see when the oxide is disturbed. The tubes are then partly filled with cold freshly boiled water, the pairs balanced and once more centrifugated for two or three minutes, after which the wash water is poured off as before. The cuprous oxide may now be determined in either of two ways, both of which are satisfactory.

Colorimetric determination of cuprous oxide.[6] The sediment of cuprous oxide is dissolved in the smallest possible amount of concentrated nitric acid (2 to 10 drops), and is then diluted with ammonia water (1 part strong NH_4OH to 5 parts water) to such a volume that the blue color is roughly about the same as the standard. Cylinders (10 cc. to 50 cc.) may be used for this dilution, though of course the reading of the volume should be accurately made.[7] The unknown is then read against the standard copper solution, the latter set at 20 mm. in a Duboscq colorimeter.

[6] The colorimetric determination of copper has been used for years in metallurgical assaying (Heath: *Journ. Amer. Chem. Soc.*, xix, p. 24, 1897), but its use does not appear to have become popular in sugar analysis. Sahli (*Lehrbuch. d. klin. Untersuchungs Methoden*, 1902) determined colorimetrically the copper oxide from sugar reduction after dissolving in nitric acid, but he used no colorimeter and obtained only approximate results. Ocrum (*Zeitschr. f. anal. Chem.*, xliii, p. 356, 1904) worked out the conditions more accurately, but both he and Sahli appear to have used only the green acid color. I am not aware of the use of the Sahli method by other workers.

[7] I have found it very convenient to have the centrifuge tubes graduated at 10, 20, 30 and 40 cc. (if properly etched, the marking does not weaken the resistance to the boiling water), the appropriate dilution then being made in the tube without transfer. The graduation of the tubes should be calibrated before use.

The standard ammoniacal copper solution contains 5 mgm. of copper in 10 cc. and is prepared by dissolving 9.82 grams of crystalline copper sulphate in 500 cc.; 10 cc., of this solution is diluted to 100 cc. with 1 to 5 ammonia, which gives a solution that may be easily and accurately read in the colorimeter. I find it best to set the standard at 20 mm., though from 10 to 30 mm. it reads almost equally well. The unknown should be between the limits of from half to twice the color of the standard; within these limits the dilution is without much influence. The amount of copper in the unknown is found from the following formula:

$$\frac{St. \times 5 \times v}{R \times 10} = \text{mgm. Cu. found}$$

in which $St.$ = reading of standard, R = reading of unknown, and v = volume of unknown.

This method has been found quick and convenient, though not especially more so than the Bertrand titration as described below. The results agree within 2 or 3 per cent.

Determination of copper by Bertrand titration. The washed cuprous oxide contained in the centrifuge tube is dissolved by adding about 0.5 cc. of a strong ferric sulphate sulphuric acid solution,[8] and is immediately titrated in the centrifuge tube with $\frac{N}{20}$, $\frac{N}{50}$ or $\frac{N}{100}$ potassium permanganate, depending upon the amount of cuprous oxide present. The small amount of liquid in the tube permits a sharp end-point to the titration even with very dilute permanganate. The permanganate should be freshly prepared from a standardized $\frac{N}{10}$ stock solution. One cc. of $\frac{N}{10}$ permanganate is equivalent to 6.36 mgm. of copper.

Correction and calculation. Practically all preparations of Fehling's solution deposit small amounts of cuprous oxide when heated. Blank determinations of 20 cc. of Fehling's solution with 20 cc. of water heated in a boiling water bath for ten minutes usually yield the equivalent of about 1 mgm. of copper. Such blank determinations must be made on the solution used and the amount subtracted as a correction.

For the conversion of the result in terms of copper into milligrams of glucose the following table is used. The table has been

[8] Ten per cent ferric sulphate in 25 per cent sulphuric acid, to which is added just enough permanganate to oxidize any ferrous salt which may be present.

prepared from the results of a large number of determinations of pure glucose solutions. The amount of copper found, *minus the correction*, divided by the corresponding factor, equals milligrams of glucose in the blood represented by the amount of filtrate used. For example, 21 cc. of blood filtrate, representing 3 cc. of blood, gave a titration of 5.5 cc. $\frac{N}{50}$ permanganate.

5.5 × 1.27 = 6.99 − 1.2 (Correction) = 5.79.
5.79 ÷ 2.19 = 2.64 mgm. glucose or 0.088 per cent in the blood.

TABLE I.

Standard copper: glucose values.[9]

20 cc. of sugar solution plus 20 cc. Fehling's solution, heated 10 minutes in a boiling water bath. Mgm. Cu ÷ divisor = mgm. glucose.

MGM. COPPER	DIVISOR	MGM. COPPER	DIVISOR	MGM. COPPER	DIVISOR
0.7	1.50	6.0	2.19	20.0	2.06
1.0	1.60	7.0	2.18	25.0	2.04
1.5	1.70	8.0	2.17	30.0	2.03
2.0	1.80	9.0	2.16	35.0	2.01
2.5	1.90	10.0	2.15	40.0	2.00
3.0	2.00	12.0	2.14	50.0	2.00
3.5	2.10	14.0	2.12	60.0	1.99
4.0	2.20	16.0	2.10	80.0	1.98
5.0	2.20	18.0	2.08	100.0	1.97

Methyl alcohol as the protein precipitant. I recently[10] described and demonstrated a method for blood sugar in which methyl alcohol was used for the precipitation of the proteins. The technique was simple and convenient and yielded concordant results, but had to be abandoned because it was found that it did not accomplish complete extraction of the sugar. When sugar was added to fresh blood, the added amount was determined accurately and it was therefore at first thought that the results by the method were correct; but it later appeared that this was not so because a portion of the sugar originally present is held in the precipitated protein. On directly comparing the results after

[9] Except below 4 mgm. of copper, these values are very close to those of Moeckel and Frank, (*Zeitschr. f. physiol. Chem.*, lxv, p. 325).

[10] Before the American Society of Biological Chemists, Philadelphia, December, 1913.

methyl alcohol extraction with those after colloidal iron precipitation of the proteins, it was found that the latter results are uniformly higher. We had already made a large number of observations with the use of methyl alcohol and it was therefore important to know which results to accept. The results in Table II indicate the differences obtained by the two methods.[11]

TABLE II.

Comparison of results of methyl alcohol and colloidal iron precipitation.

	METHOD	EQUIVALENT OF BLOOD USED IN REDUCTION	NET CU. FOUND	GLUCOSE	PER CENT GLUCOSE IN BLOOD
		cc.	*mgm.*	*mgm.*	
Fresh defibrinated dog blood.....	Iron	8.91	35.5	17.5	0.196
	Iron	8.50	33.6	16.6	0.195
	Iron	9.30	37.6	18.6	0.200
	Iron	9.08	37.2	18.4	0.202
	Iron	8.20	32.6	16.1	0.196
	Iron	9.22	36.7	18.2	0.198
	Methyl alc.	9.01	19.1	9.2	0.103
	Methyl alc.	12.80	26.0	12.7	0.100
	Methyl alc.	8.94	20.0	9.7	0.108
Dog plasma...	Iron	9.37	15.4	7.3	0.078
	Methyl alc.	7.50	7.4	3.4	0.045
Fresh defibrinated dog blood.....	Iron	8.34	27.5	13.5	0.162
	Iron	8.34	27.7	13.6	0.163
	Methyl alc.	8.40	18.3	8.8	0.105
	Methyl alc.	4.56	10.0	4.65	0.102
Serum of same blood	Iron	6.66	33.8	16.7	0.250
	Iron	6.66	31.3	17.0	0.255
	Methyl alc.	8.80	32.8	16.2	0.184

[11] In the methyl alcohol procedure 10 cc. of blood were added to 50 cc. of the alcohol, and the mixture diluted with more alcohol to 100 cc. An aliquot part of the filtrate, acidified with acetic acid, was evaporated to dryness on a water bath, the film washed with a little ether, dissolved and rinsed into a centrifuge tube and the reduction determined as above described. In the iron method 10 cc. of blood were diluted with 80 cc. of water, 50 cc. of colloidal iron solution was added, and the colloids were precipitated by 10 cc. of saturated sodium sulphate solution. After centrifugation and filtration, an aliquot of the filtrate (about 100 cc., 15 cc. = 1 cc. blood) was acidified and evaporated. The residue was rinsed into a centrifuge tube, diluted to 20 cc. and its reduction determined as above.

The questions involved proved less simple than expected and a good deal of time has been spent without reaching altogether satisfactory conclusions. The following points of difference, however, appear established. When blood is run into methyl alcohol, the cells are "fixed" and the cell-sugar is not extracted, unless the cells have been previously laked; the cells are laked and the cell-sugar is extracted during the colloidal iron precipitation. During the evaporation of the aqueous filtrate from the iron precipitation acidified with acetic acid there is a slight increase in its reducing power, corresponding to Lépine's *virtuel* sugar; there is little or no increased reducing power as a result of the evaporation of the methyl alcohol solution. And finally, the methyl alcohol does not accomplish the complete extraction of the plasma sugar, a part being retained by the precipitate; the aqueous filtrate from the iron precipitation on the other hand appears to contain all the sugar.

All of the reducing substance contained in the latter filtrate is readily fermentable by yeast and is therefore presumably sugar. The evidence is all in favor of the colloidal iron precipitation and the use of the methyl alcohol has therefore been abandoned for this purpose.[12]

Is the cuprous oxide completely precipitated? In the course of my experiments I attempted as others have done, to test the completeness of extraction of the blood sugar by using old blood (which stood at room temperature for several days and which had lost its reducing power), to which was added a known amount of pure glucose. This test cannot be used for the reason that low results are frequently obtained with such mixtures by any method.

[12] According to some preliminary experiments it is probable that amino acids and other similar substances are also incompletely extracted by methyl alcohol, and that the Folin-Denis technique for the determination of non-protein-nitrogen, etc., in blood would be improved by the substitution of the Michaelis-Rona colloidal iron precipitation for the methyl alcohol.

For the extraction of sugar, ethyl alcohol, acetone, mixtures of the two, and mixtures of each with methyl alcohol and with water are all wholly unsatisfactory. Ethyl alcohol especially, extracts but a small portion of the sugar and it is difficult to see how it could have been used so frequently in the past in blood-sugar determinations (Bang: *Biochem. Zeitschr.*, vii, p. 327, 1907). Certainly the results obtained with its use are unreliable,

Low results are obtained even if one adds a known amount of glucose to the *filtrate* from the colloidal iron precipitate immediately before the treatment with Fehling's solution; some substances are present which hold a part of the cuprous oxide in solution. The importance of this is that it raises the question whether the *filtrates from fresh blood contain such substances, for if such be* the case, the results based upon the determination of the precipitated cuprous oxide are too low. It is known that the results by the older Bang method are higher than those by the cuprous oxide methods (Moeckel-Frank); but the amount of cuprous oxide precipitated is usually supposed to indicate the amount of sugar more accurately than does the "total reduction."[13]

The protein-free filtrates of *fresh* blood probably do not hold cuprous oxide in solution. This is indicated by the fact that when such filtrates are fermented by yeast and glucose is added *after* fermentation, although low results are obtained for the added sugar, the amount of cuprous oxide held in solution is no greater than the amount held in solution by a suspension of an equal amount of yeast incubated alone. The low results with fermented blood extracts containing added sugar are due to incomplete precipitation of cuprous oxide, some being held in solution by something extracted from the yeast. And since we can account for the low results with fermented *fresh* blood filtrates, as being due to the yeast, it is reasonable to conclude that the fresh blood

except possibly for comparative purposes. The following results were obtained on one sample of defibrinated dog blood with the use of the precipitants mentioned; the technique was that already described for methyl alcohol.

	Method	*Sugar found, percent.*
I	Colloidal iron	0.086
	Methyl alcohol	0.078
	Methyl alcohol and acetone, equal parts	0.073
	Acetone	0.099
	Ethyl alcohol	0.094
	Ethyl alcohol and acetone, equal parts	0.099
II	Colloidal iron	0.119
	Methyl alcohol, 90 per cent	0.098
	Methyl alcohol, 80 per cent	0.106
	Methyl alcohol, 70 per cent	0.114
	Methyl alcohol, 60 per cent	0.115
	Methyl alcohol, 50 per cent	0.118

[13] Frank and Bretschneider: *Zeitschr. f. physiol. Chem.*, lxxi, p. 157, 1911.

filtrates before fermentation did not contain interfering substances, and that the results from the determination of the cuprous oxide are correct.[14] I know of no more satisfactory way of deciding the point, though I have devoted some time to it. The following experiments with beef blood illustrate the above considerations. Similar experiments with dog blood led to practically the same results.

Twenty-five cc. of fresh beef blood, diluted with 104 cc. of water, was quickly heated just to boiling and a few drops of acetic acid added. Then 25 cc. of colloidal iron solution was added and later about a gram of powdered sodium sulphate. The mixture was cetrifugated and filtered. Six cc. of the filtrate represented 1 cc. of blood. A portion of the filtrate (75 cc.) was treated with a half cake of baker's yeast and incubated over night. The yeast was removed by adding 7.5 cc. of colloidal iron and filtering. Portions of this fermented filtrate with and without added glucose were used for the sugar determination as already described. Similar determinations were made on filtrates from yeast in water alone.

	Cu reduced *mgm.*	Cu held in solution *mgm.*
Filtrate equivalent to 3 cc. blood before fermentation	12.35	
Same amount of filtrate after fermentation	0.00	
Same amount of filtrate after fermentation + 5.0 mgm. of glucose	7.80	3.4
	8.10	3.1
5. mgm. of glucose alone	11.20	
Autolysed yeast filtrate	0.00	
Autolysed yeast filtrate + 5. mgm. glucose	7.58	3.6

SUMMARY.

Technique is described for the determination of sugar in 5 cc. of blood. The results from whole blood represent the sum of plasma and cell sugar, and are believed to be correct within 5 per cent.

The importance of the conditions under which the blood is drawn is emphasized.

The use of methyl alcohol is not suitable for the extraction of sugar.

[14] Lesser (*Biochem. Zeitschr.*, liv, p. 252, 1913) appears to find that this condition does not hold for the blood of frogs and turtles.

ON THE NORMAL LEVEL OF BLOOD-SUGAR OF THE DOG.

By PHILIP A. SHAFFER.

(From the Laboratory of Biological Chemistry, Washington University, St. Louis.)

(Received for publication, August 28, 1914.)

According to the numerous observations in the literature, the normal variations in the concentration of sugar in the blood of the dog are very great. The subject has recently been reviewed by two of the active investigators in this field, one of whom, Bang, reaches the conclusion that the variations are so great that the dog is little suited for investigations concerning glycaemia.[1] Bang's summary of the newer analyses by presumably satisfactory methods, shows results between 0.04 and 0.22 per cent. Macleod[2] cites, as representing normal values, results of his own and of other workers which vary between 0.073 and 0.219 per cent. It would seem fairly evident that if these different results really represent the strictly normal variations in the blood-sugar of this animal, the dog is, as Bang points out, poorly suited for work having its basis in the determination of an increase above the normal blood-sugar. Yet dogs have been used very extensively in work of this character; and in many cases, when positive findings have been obtained, as a result of the factor being investigated, the differences in values have been little if any greater than the above supposedly normal variations.

The true situation is, I believe, shown by a series of experiments, three of which are reported in this paper. The blood of dogs as well as of other laboratory animals, which has been used for almost all of the determinations in the past, has been drawn after cutting down and laying bare a blood vessel, with or without anaesthesia, or after otherwise causing some pain or fright, and

[1] Bang: *Der Blutzucker*, Wiesbaden, 1913, p. 31.

[2] Macleod: *Diabetes*, London, 1913, pp. 28 and 30.

the results obtained represent not at all the *normal* value of blood-sugar for the animal, but merely varying degrees of hyperglycaemia caused by fright, pain or similar nervous factors. Results obtained under such conditions undoubtedly may be *natural* or *physiological* values, but they cannot be classed as *normal*, in the way that term is usually interpreted. The *normal* blood sugar of the dog, that is when the animal is not in a state of excitement or pain, is surprisingly constant and is correctly represented not by the average of the results previously published, but by the lowest so far observed.

Hirsch and Reinbach[3] and Loewy and Rosenberg[4] have already pointed out the marked "Fesselungshyperglycaemia" which is produced in rabbits, and the latter authors show that also with dogs lower results (0.10 to 0.15 per cent) are obtained with local anaesthesia than without (0.10 to 0.22 per cent). Twenty to 50 cc. of blood was drawn from the carotid or femoral vein after incision. They concluded that the "Fesselungshyperglycaemia" is less marked in the dog than in the rabbit, but that the sugar values in blood drawn without anaesthesia are unreliable. Their supposedly normal results are, however, too high.

Scott[5] in a recent paper has made similar observations for the cat, and states that when the blood-sugar is to be determined "any work which would otherwise involve pain must be accompanied by an anaesthetic." But the administration of a general anaesthetic like ether or chloroform, itself causes a marked rise of the blood-sugar. Scott himself found after ether anaesthesia from 0.086 to 0.302 per cent as compared with from 0.059 to 0.096 per cent after sudden decapitation. He cites results from 0.11 to 0.31 per cent in blood from the carotid without anaesthesia and from 0.154 to 0.355 per cent in the carotid from blood drawn under light anaesthesia.

The only normal values for the blood-sugar of the dog which I have found to agree with my results are those of Embden, Lüthje and Liefmann[6] who fully appreciated the danger of exciting the animals and who drew the blood as I have done, by direct puncture

[3] *Zeitschr. f. physiol. Chem.*, lxxxvii, p. 122, 1913.
[4] *Biochem. Zeitschr.*, lvi, p. 115, 1913.
[5] Scott: *Amer. Journ. of Physiol.*, xxxiv, p. 271, 1914.
[6] *Hofmeister's Beiträge*, x, p. 265, 1907.

of the jugular vein through the skin. Their results vary from 0.057 to 0.088 per cent[7] or about half that usually accepted as the normal percentage. I fail to understand why these results have not received more consideration, since they undoubtedly represent the closest approach to really *normal* values to be found in the literature. Oppler and Rona[8] have also published fairly correct values, though they made an incision through the skin, probably causing slight pain, before drawing the blood; and their results are somewhat higher (in dogs from 0.072 to 0.096 per cent).

In the following experiments the first blood samples, designated the (normal) values, were drawn as described in the preceding paper, by inserting a sharp syringe needle, attached by a short rubber tube to a pipette, directly through the skin into the jugular vein or into the femoral artery. There was no struggling or evidence of pain or fright, the animals in most cases being ac-

Experiment I. Young dog, weight about 7 kgm.

NO.	TIME		PER CENT SUGAR IN BLOOD
1	12:00	10 cc. blood drawn from jugular (normal)	0.037 0.034
2	12:05	10 cc. blood drawn from femoral artery (normal)	0.047 0.045
	12:10	Anaesthetized with ether, with the usual struggling. Anaesthetic continued.	
3	12:20	Made incision and bled 10 cc. from carotid	0.090 0.082
4	12:25	10 cc. blood drawn from jugular as before	0.081 0.082
5	12:30	Bled several hundred cc. rapidly from carotid. Samples taken of this mixed blood	0.081 0.082
6	12:35	Opened abdomen and drew 10 cc. from vena cava	0.107 0.109
7	12:40	Drew 10 cc. blood from aorta	0.123 0.121
8	12:45	Continued bleeding from carotid.	
	12:55	When about exsanguinated collected 10 cc. from carotid	0.126 0.123

[7] At 25° to 30° C. temperature of the surrounding air. Somewhat higher results were found on exposure to cold.

[8] *Biochem. Zeitschr.*, xiii, p. 120, 1908.

Experiment II. Dog, weight 5.6 kgm.. Good condition. Fed 18 hours before experiment.

NO.	TIME		PER CENT SUGAR IN BLOOD
1	11:05	Drew 10 cc. blood from jugular (normal).....	0.022 0.018
2	11:10	Drew 10 cc. blood from femoral artery (normal)..	0.020 0.019
3	11:13	Drew 10 cc. from jugular as before (normal)...	0.018 0.018
	11:17	Anaesthetized with ether. The usual struggling. Continued anaesthetic.	
4	11:30	Drew 10 cc. blood from jugular as before, no incision.....	0.095 0.093
	11:33	Made incision and laid bare carotid and jugular.	
5	11:40	Drew 10 cc. from carotid.....	0.171 0.159
6	11:44	Drew 10 cc. from jugular.....	0.168 0.174
	11:47	Continued bleeding from carotid.	
7	11:52	Collected 10 cc. from carotid after great hemorrhage.....	0.245 0.240
8	12:00	Last 15 cc. from carotid.....	0.320 0.320

Experiment III. Dog, weight 8 kgm.

NO.	TIME		PER CENT SUGAR IN BLOOD
1	1:15	Bled 10 cc. from jugular. Dog quiet in cage (normal).....	0.043 0.049
	1:18	Began five minute exercise by running up and down stairs.	
2	1:25	Immediately after exercise, 10 cc. from jugular (normal).....	0.049
3	3:00	Quiet in cage for 1½ hours. 10 cc. from jugular (normal).....	0.049 0.043
4	3:10	Femoral artery (normal).....	0.051 0.058
	3:12	Anaesthetized with ether. Made incision and laid bare carotid and jugular. Continued anaesthetic.	
5	3:35	Bled 10 cc. from jugular.....	0.120 0.122
6	3:38	Bled 10 cc. from carotid.....	0.130 0.131
7	3:40	Continued bleeding from carotid until nearly exsanguinated. This mixed blood.....	0.140 0.141
8	3:45	Last blood from carotid.....	0.138 0.129

customed to the operation. The remaining samples were drawn as stated in the protocols. In each case 10 cc. of blood were run at once into 52 cc. of water and heated to boiling, the rest of the determination being carried out according to the method described in the preceding paper. The cuprous oxide formed from the reduction by filtrate equivalent to 3 cc. of blood, was determined by the Bertrand titration.

I have determined by the procedures already described the normal blood-sugar of about twenty different dogs, in some instances over periods of several weeks and under different conditions as regards food and state of nutrition. The results varied between 0.02 and 0.065 per cent and these values may therefore be taken as being more nearly the normal limits of the concentration of the blood-sugar of the dog. The results from a few animals are given below; about a week elaspsed between these determinations:

		Per cent
Dog 1	Fed	0.026
	Fasting	0.043
	Fasting	0.043
Dog 2	Fed	0.041
	Fasting	0.047
	Fasting	0.054
Dog 3	Fed	0.048
	Fasting	0.059
	Fasting	0.051
Dog 4	Fed	0.045
	Fasting	0.053
	Fasting	0.053
Dog 5	Fed	0.053
	Fed	0.059

These results and those in the three experiments given show very definitely that the concentration of sugar in the blood of normal dogs, when the animals are free from excitement or pain, is in the neighborhood of 0.05 per cent or about one-half or one-third the values usually accepted as normal. The higher results obtained after anaesthesia are comparable with those usually obtained in the past and represent varying degrees of emotional

hyperglycaemia brought about by the procedure of drawing the blood.[9] The level of the blood-sugar of dogs, rabbits and cats appears to be quickly and markedly affected by emotional states, and the same is doubtless true also of man;[10] there is no reason to suppose it is true only of these species. Certain physiological aspects of this phenomenon have been considered by Cannon,[11] but its practical bearing upon the results of many investigations concerning experimental hyperglycaemia has perhaps not been sufficiently taken into account. If one accepts as the normal level a value which really represents a hyperglycaemia, it would appear probable that the effect of at least some factors will be obscured. On the other hand it is probable that the great susceptibility of laboratory animals to emotional hyperglycaemia introduces a serious obstacle into the conduct of such work. Whether this increase in blood-sugar can be avoided in work requiring surgical manipulation, and the obstacle thus removed, remains to be determined.

[9] Whether the effect is that of the anaesthetic itself is not certain, but it is more likely due to fright during its administration.

[10] Folin has recently pointed out the existence of transitory glycosuria in normal men and women following even mild emotional strain.

The values already published for the normal blood-sugar of man agree with my observations (from 0.06 to 0.11 per cent). Human blood for analysis is almost always drawn from a superficial vein without incision and without causing pain.

[11] Cannon: *Amer. Journ. of Physiol.*, xxxiii, p. 357, 1914.

PARAHYDROXYPHENYLETHYLAMINE, A PRESSOR COMPOUND IN AN AMERICAN MISTLETOE.

By ALBERT C. CRAWFORD and WALTER K. WATANABE.

(*From the Division of Pharmocology, Stanford Medical School, San Francisco.*)

(Received for publication, September 3, 1914)

In 1905[1] one of us called attention to the fact that the intravenous injection into dogs of extracts of *Phoradendron flavescens* (mistletoe) would cause a rise in blood pressure with acceleration of the heart rate and in 1911[2] described a method for isolating one of the pressor compounds as an oxalate, and gave provisionally $C_7H_{11}N$ as its empirical formula. However, it was found that different samples gave somewhat different determinations; hence some impurities were suspected to be present. Recently, we have returned to this work and have found that a benzoate made from this oxalate, when crystallized from hot 70 per cent ethyl alcohol, melted at 170°C., a melting point which corresponded to that of dibenzoyl-*p*-hydroxyphenylethylamine, and when mixed with an equal amount of the benzoate of the synthetic compound the melting point remained the same. Nitrogen determinations of this benzoate gave 4.10 and 4.14 per cent. The calculated percentage of nitrogen in dibenzoyl-*p*-hydroxyphenylethylamine is 4.06. This benzoate was hydrolyzed with 20 per cent HCl at 140°C.[3] and then converted into an oxalate. This exerted quantitatively the same pressor action as the oxalate prepared from synthetic *p*-hydroxyphenylethylamine. A picrate prepared from the oxalate melted at 200°C, the same temperature at which the picrate of *p*-hydroxyphenylethylamine melted.[4] An oxalate was prepared from the aqueous extract

[1] *Amer. Journ. of Phormocy*, 1905, p. 493.
[2] Crawford: *Journ. Amer. Med. Assoc.*, lvii, p. 865, 1911.
[3] Barger: *Trans. Chem. Soc.*, xcv, p. 1123, 1909.
[4] Barger: *loc. cit.*

of the crude plant[5] by making it alkaline with NaOH and shaking with ether, in order to remove any possible phenylethylamine or non-phenolic bases, then neutralizing with HCl, rendering alkaline with Na_2CO_3, shaking with ether and precipitating the base with an anhydrous ethereal solution of oxalic acid. This oxalate melted at 204°C., as did the oxalate of synthetic *p*-hydroxyphenylethylamine.

From 100 cc. of the fluid extract of mistletoe about 150 mgm. of the oxalate was obtained by our original method,[6] *i.e.*, 1 cc. would contain about 1.5 mgm. but 1 cc. of the fluid extract raised the blood pressure of dogs, with cut vagi, more than this amount of *p*-hydroxyphenylethylamine oxalate, hence we infer, as originally suggested, that there are other pressor compounds, but as yet we have not proved the presence of phenylethylamine or isoamylamine, compounds which are associated with *p*-hydroxyphenylethylamine in ergot extracts. The alkaline distillate in which phenylethylamine should appear, if present, is inactive.

In dogs preceding the rise in blood pressure from the injection of fluid extract of mistletoe, there is a temporary fall in blood pressure, even if the vagi are cut. In ergot extracts the depressor action on blood-pressure is traced to acetyl-choline and perhaps choline.[7] On following Ewins' method for isolating acetyl-choline from ergot we obtained a solution which gave a temporary fall, without a rise, but as yet have not positively identified acetyl-choline in mistletoe.

[5] The ground mistletoe was sent to us by Parke, Davis & Co., of Detroit, Mich. All melting point readings are uncorrected.

[6] Crawford: *loc. cit.*, 1911.

[7] Ewins: *Biochem. Journ.*, viii, p. 44, 1914; Dale: *Journ. of Physiol.*, xlviii, p. iii, 1914.

IMMUNIZATION AGAINST THE ANTICOAGULATING EFFECT OF HIRUDIN.

By MIGUEL VERA and LEO LOEB.

(From the Department of Pathology, Barnard Free Skin and Cancer Hospital, St. Louis.)

(Received for publication, September 3, 1914.)

In former experiments we found that various substances, among them hirudin, injected intravenously into mice inhibited the growth of mouse tumors.[1] We found, furthermore, that in the course of the injections of the solutions of colloidal copper and of hirudin, immunity was produced against the effect of these substances, the rate of growth of the tumors serving as an indicator of the degree of immunity reached. We were able to show that this immunity was of a twofold origin; namely, it had its source, first, in a process of immunization taking place somewhere in the organism of the injected animal; and, second, in changes taking place in the tumor cells themselves as the result of the injections, the tumor cells themselves becoming immune against the action of these substances. This latter kind of immunity could be transmitted through several generations of tumor cells.

In order to obtain a further insight into the first component of this process of immunization, we decided to study the immunity produced through injection of hirudin into mice, using the effect of repeated injections on the production of substances affecting the coagulation of the blood as an indicator.

It has been found in former experiments by Ledoux[2] that a few injections of hirudin into rabbits do not produce immunity. Wendelstadt[3] believed that he produced an antibody against hirudin. After numerous intraperitoneal or subcutaneous injections of hirudin into rabbits, he obtained an antibody which inhibited the

[1] *Journ. Am. Med. Assn.*, lx, p. 1857, 1913.

[2] Ledoux: *Arch. de biol.*, xiv, p. 63, 1895.

[3] Wendelstadt: *Arch. internat. de pharmacodynamie*, ix, p. 407, 1901.

coagulation of the hirudin- and also of histon-blood in rabbits. It was without effect on liquor pericardii, on horse plasma and oxalate and $MgSO_4$ plasma. The blood of a normal animal coagulated as rapidly as the blood of an immunized one. Heating to 56° and 60° diminished the activity of the antibody. It was possible to obtain an antibody against this hirudin antibody by injecting the immune serum into rabbits or rats. This antibody inhibited the coagulation of normal blood. Cowie[4] also obtained an antibody against hirudin in rabbits. The effect of this antibody could not only be demonstrated in hirudin blood mixture, but also in mixtures of hirudin with solutions of fibrinogen and of hydrocele fluid. He suggests that the effect of the immunization consists in new production of thrombin or of thrombogen.

In our experiments we immunized mice instead of rabbits and used intravenous injections. We gave an injection every day and used increasing doses of hirudin; 0.5 cc. of the solution was injected each time. On the first day a 0.025 per cent, on the second day a 0.05 per cent, on the third day a 0.1 per cent, on the fourth day a 0.25 per cent, on the fifth day a 0.33 per cent, on the sixth day a 0.5 per cent, on the seventh day a 0.63 per cent solution of hirudin in 0.85 per cent NaCl solution was used. In order to test the effect of hirudin on the blood of the immunized animals, usually the dose given on the fourth or fifth day, sometimes a stronger solution, was used. The blood of mice having received a series of injections of hirudin will be referred to as "prepared blood," the serum obtained from such blood as "prepared serum," and the mice injected as "prepared mice."

In order to test the effect of hirudin on the coagulation of blood *in vitro*, equal amounts of a 0.022–0.08 per cent of hirudin in 0.85 per cent NaCl solution and of blood were used. The blood was collected from the vessels of the neck. It was important that the blood should flow directly and rapidly into the test-tube.

We tested the acquired immunity in mice against the anticoagulating action of hirudin in various ways, *in vivo* as well as *in vitro*.

(*a*) We injected hirudin in normal as well as in prepared mice (having received a series of injections of hirudin), and at various

[4] Cowie: *Journ. of Med. Research*, xxiv, p. 497, 1911.

periods after the test injection of hirudin we withdrew the blood of the animals and observed the coagulation time. In the prepared animals the coagulation time should be shorter than in the controls.

(*b*) In order to test whether a certain amount of hirudin was in some way inactivated and prevented from further action on coagulation through the blood of prepared mice, we collected blood from normal and from prepared mice separately in a solution of hirudin. Both kinds of blood were then separately injected into normal mice, and a short time later blood was withdrawn from these animals. In case a greater amount of hirudin had been inactivated through the blood of the prepared mice, the blood of mice injected with the hirudin plus the blood of the prepared mice should coagulate more quickly than the blood of mice injected with the mixture of hirudin and the blood of control mice.

(*c*) We collected the blood of prepared and of normal mice in solutions of hirudin *in vitro* and observed the coagulation time. The blood of the prepared animals should coagulate more rapidly than the blood of normal mice in the hirudin solution.

(*d*) We obtained the serum from the blood of normal mice and of prepared mice and compared the action of both kinds of sera on hirudin blood. The serum of the prepared mice should cause a more rapid coagulation in hirudin blood than the control serum.

Our results were as follows:

1. *Method A*. If we inject hirudin into a prepared animal and withdraw the blood five minutes after the injection, it coagulates very much more rapidly than the blood of a control animal. In an experiment the blood of the prepared animal coagulated in eleven to eighteen minutes, while the blood of a control mouse was not yet coagulated five hours later. In these experiments the blood was withdrawn twenty-four to thirty-four hours after the last immunizing injection. Four or eight hours after the last immunizing injection the result is already definite. After four preparatory injections the presence of the immunizing substance can already be demonstrated. It becomes more marked after six to seven injections and seems to be still more marked after ten and twelve injections, when even after injection of hirudin solution No. 6 the blood of the prepared animals coagulates rapidly.

We are, therefore, able to demonstrate the presence of an acquired immunity within five days after the first injection.

If, instead of five minutes after the test injection of hirudin the blood is withdrawn thirty to sixty minutes afterwards, no difference can be observed between the coagulation of the blood of the prepared and control animals, the remaining hirudin being eliminated from the blood of prepared as well as of the control animals during the interval between the test injection and the withdrawal of the blood.

2. *Method B.* If we receive the blood of a prepared mouse in a solution of hirudin, a much greater part of the hirudin is neutralized than if we receive the blood of a normal animal in hirudin. If we inject each mixture separately into the circulation of normal mice, withdraw the blood of the injected animals five minutes after the injection, the blood of the one injected with a mixture of hirudin and prepared blood coagulates much quicker than the blood of the animal injected with normal blood-hirudin mixture. We may therefore conclude that some substance in the blood of the prepared animals inactivated in some way the hirudin mixed with it. In an experiment, for instance, the blood of five mice injected with a mixture of hirudin and the blood of prepared mice coagulated in 6, 6, 7, 6 and 12 minutes, respectively, while the blood of six mice injected with a mixture of hirudin and the blood of normal mice coagulated in 4 hours 40 minutes; 4 hours 51 minutes; 3 hours 51 minutes; 2 hours 31 minutes; 2 hours 54 minutes and 4 hours 31 minutes.

3. The coagulation time of the blood of normal and of prepared mice (the blood of the latter being withdrawn twenty-five to thirty hours after the last injection of hirudin) is approximately the same. Average of coagulation time of the blood for 22 prepared mice: 2 minutes 54 seconds; average for the blood of 22 controls: 2 minutes 40 seconds.

4. *Method C.* If we collect the blood of prepared mice in a solution of hirudin, the blood coagulates more rapidly than the blood of control mice; while in the prepared mice the blood coagulates usually in from 9 to 27 minutes; in controls the coagulation time varies between 3 hours and 30 minutes and one to two days. In these experiments the prepared mice had usually received six or seven injections of hirudin and the blood was usually withdrawn twenty-four to forty hours after the last injection.

5. If we withdraw the blood of prepared animals eight to nine instead of one to two days after the last—sixth or seventh—injection of hirudin, immunity is still present in most cases, the blood coagulating very much more rapidly in prepared than in control animals, but in some animals the immunity is no longer very marked or has disappeared altogether. The results are therefore not quite so regular as when the test is performed twenty-four to thirty hours after the last injection. In one experiment the blood of the prepared animals coagulated in 12 minutes; 20 minutes; 17 minutes; 4 hours 30 minutes; in the fifth it had not yet coagulated in 12 hours. The blood had been withdrawn nine days after the last injection. In four controls the blood had not yet coagulated in 10 hours.

6. While in our first experiments we had obtained distinct results if the blood was withdrawn twenty-four hours after the last preparatory injection, in some later experiments in which we used a different (probably a weaker) sample of hirudin, immunity was either not present one day after the last injection, or it was relatively weak, became more pronounced on the second and reached a maximum on the third day, after which time it began again to decline. There existed therefore in these experiments a distinct curve of the degree of immunity, which reached a maximum two to three days after the last preparatory injection.

Example. In prepared mice after 24 hours: not coagulated in 12 hours. After 48 hours: coagulated in 26 minutes. After 3 days: coagulated in 10 minutes. After 5 days: coagulated in 32 minutes.

In the control mice the blood usually did not coagulate in 24 hours, but in one lot it coagulated in from 8 to 18 hours.

7. In a series of experiments in which we used prepared tumor mice instead of prepared normal mice, we found the immunity on the first day usually to be weaker than in prepared normal mice; in some lots in which immunity was not present one day after the last injection it was demonstrable after three days. In another experiment it seemed that mice in which the tumors had been removed before the last preparatory injections had been given had developed a greater degree of immunity than mice in which the tumors had not been removed or in which it recurred rapidly after an operation; in this experiment, how-

ever, immunity had developed notwithstanding the presence of large tumors. It seems, therefore, that in all probability the presence of a tumor has to a certain extent an inhibiting effect on the rapid appearance of the immune substance. For instance, in prepared mice with large tumors the blood coagulated in 28 minutes; 7 hours 21 minutes; 32–44 hours; in prepared tumor mice in which the tumor had been removed and in which no recurrence took place during the period of observation the blood coagulated in 12 minutes; 15 minutes; 10 minutes; in a prepared and operated tumor mouse in which a recurrence of the tumor took place the blood coagulated in 1 hour 21 minutes; in not prepared control mice the blood coagulated in 24 hours; 15 hours; and in a third animal it had not coagulated in 5 hours.

8. If we add serum obtained either through defibrination or *through clotting of the blood of mice immunized against hirudin* to a mixture of hirudin and normal blood *in vitro*, the coagulation of the blood takes place much more quickly than if the serum of normal controls had been added to the mixture. For instance, in one experiment coagulation occurred in 8–30 minutes after addition of immune serum, and in 1–2 days after addition of control serum. There is a certain relation, but no direct proportionality, between the quantity of serum added and the coagulation time: there are apparently various substances present in the serum with an opposite effect on the coagulation of the blood. For instance, in one experiment:

Blood coagulates in 40 minutes with 1 drop immune serum; in 3–5 hours with 1 drop control serum.

Blood coagulates in 22 minutes with 2 drops immune serum; in 3–5 hours with 2 drops control serum.

Blood coagulates in 15 minutes with 4 drops immune serum; in 3–5 hours with 4 drops control serum.

In another experiment:

Blood coagulates in 1 hour 42 minutes with 1 drop immune serum; in 17 hours with 1 drop control serum.

Blood coagulates in 37 minutes with 2 drops immune serum; in 5–15 hours with 2 drops control serum.

9. Heating immune serum to 56°C. for 35 minutes did not decrease its coagulating power; heating it to 60°C. for 35 minutes

decreased its power somewhat, but not very markedly; while heating it to 65°C. for the same length of time caused its complete disappearance. For instance:

Immune serum heated to 56° caused coagulation in 49 minutes (1 drop serum); in 36 minutes (2 drops serum).

After heating to 60°, 2 drops immune serum caused coagulation in 25 minutes.

After heating to 65°, neither 1 nor 2 drops caused coagulation in 10 hours.

Unheated immune serum caused coagulation in 51 minutes (2 drops), in 36 minutes (1 drop); in another experiment in 71 minutes (2 drops); in a third experiment in 1 hour 5 minutes–1 hour 40 minutes (1 drop); 30 minutes (2 drops); while 1 or 2 drops control serum did not cause coagulation in 10 hours.

10. The immune substance produced in injecting mice with hirudin does not only act on a mixture of mouse blood-hirudin, but also on a guinea-pig or rabbit hirudin mixture; whether it acts relatively more strongly on mouse than on guinea-pig or rabbit blood-hirudin in mixture, whether there exists a species specificity of the immune substances produced, we cannot state definitely at the present time.

The few experiments, while suggesting that such species specificity might possibly exist, demonstrate that the difference in the action of the serum on guinea-pig, mouse and rabbit hirudin blood cannot be very great.

	A MOUSE BLOOD-HIRUDIN MIXTURE	A GUINEA-PIG BLOOD-HIRUDIN MIXTURE	A RABBIT BLOOD-HIRUDIN MIXTURE
Serum of prepared mice causes coagulation of.....	1 hr. 42 min. (1 drop)	3–5 hrs. (1 drop)	21 min. (1 drop)
	37 min. (2 drops)	37 min. (2 drops)	14 min. (2 drops)
Serum of control mice causes coagulation of.....	17 hrs. (1 drop)	17 hrs. (1 drop)	6–7 hrs. (1 drop)
	5–15 hrs. (2 drops)	5–15 hrs. (2 drops)	3 hrs. 36 min. (2 drops)

11. In another set of experiments we tried to determine whether the immune substance counteracted hirudin by directly destroying it. The possibility had to be considered that a ferment destroying the hirudin might be formed as a result of the injections; should this have been the case, a somewhat longer contact between the immune serum and hirudin should increase the effectiveness of the immune serum. We found, however, that if the blood

was added immediately after the immune serum and hirudin had been mixed, the serum was at least just as effective as when the mixture of immune serum and hirudin had been standing at room temperature for five or fifteen minutes before the addition of the blood, or it was even more effective in the former case. If the mixture of serum and hirudin had been kept on ice for fifteen minutes previous to the addition of the blood, the same effect was obtained as in the case of the immediate addition of the blood to the serum-hirudin mixture. In one experiment, for instance, the coagulation time was (if a mixture of 1 drop of immune serum and hirudin was used):

1 hour 42 minutes if the blood was added immediately to the mixture.

1 hour 49 minutes if the mixture was kept 15 minutes on ice before the addition of the blood.

3–4 hours if the mixture had been standing 5 minutes at room temperature before the addition of the blood.

7–17 hours if the mixture had been standing 15 minutes at room temperature before the addition of the blood.

Action of sodium fluoride on the blood of mice injected with hirudin.

12. If we collect the blood of a mouse in a solution of fluoride[5] instead of in a solution of hirudin, we find that the blood of those animals which have previously received a series of injections of hirudin coagulates more quickly than the blood of animals which had not received similar injections, when collected in the same solution. In some of the experiments the effect of the immunization is as marked in fluoride as in hirudin; in others, however, it is more marked in hirudin. It is necessary to use a definite amount of fluoride in which to collect the blood in order to make this effect evident. If this amount of fluoride is exceeded, the immune as well as the control bloods are incoagulable. In one experiment, for instance, the blood of prepared mice (previously injected with hirudin) coagulated in a solution of NaF in 40 minutes and in 22 minutes; in another experiment the blood of one prepared mouse coagulated in 3 hours 29 minutes; the blood of another had not yet clotted after 12 hours; while the blood of control mice was in

[5] A stock solution of 0.6 per cent NaF was used.

all cases still uncoagulated after 12 hours. This immunity against NaF is present twenty-four hours, and may still be present 9 days after the last injection of hirudin.

We found that in tumor mice also which had previously received injections of hirudin a relative immunity against sodium fluoride was present when the blood of the mice injected with hirudin was received in a solution of fluoride; but here again there were some mice which did not show any immunity. For instance, the blood of two prepared tumor mice coagulated in 8–9 hours, and in another experiment in 5 hours 25 minutes and 6–7 hours, respectively; while the blood of not prepared normal mice was in every case still uncoagulated after 24 hours.

A mixture of the blood of normal rabbit and fluoride coagulated after addition of the serum of prepared mice in 1 hour 20 minutes (1 drop); 50 minutes (2 drops); 35 minutes (4 drops); while after addition of control mouse serum the same blood fluoride mixture coagulated in 2–4 hours (1 drop); 1 hour 7 minutes (2 drops); 50 minutes (4 drops). In another experiment the mixture of normal mouse blood and fluoride coagulated in 1 hour 24 minutes with 2 drops of prepared serum and in 1 hour 52 minutes with 2 drops of control serum.

In some experiments in which we compared the action of serum of prepared and of control mice on a mixture of normal mouse or rabbit blood and fluoride, we found the immune serum to be in almost all experiments somewhat more active than the control serum; but the difference between normal and immune serum in their effect upon fluoride blood was much less marked than the difference in the coagulation time of the blood of normal and of prepared mice when received directly in fluoride solution. In some experiments there was hardly any difference between the serum of normal mice and that of mice which had received a series of hirudin injections in the effect upon a mixture of normal blood and fluoride. A slight immunity was also noticeable if we substituted rabbit blood for the mouse blood in the blood-fluoride mixture. On the whole, therefore, the immunity of mice which had received injections of hirudin was not as great towards fluoride as towards hirudin. There exists, therefore, a relative specificity of the immunity acquired through a series of injections of hirudin.

Action of citrate on the blood of mice injected with hirudin.

13. If we receive the blood of mice immunized against hirudin and of normal control mice in a solution of sodium citrate,[6] we find that the blood of normal controls coagulates *at least as rapidly* as the blood of prepared mice. There is, therefore, no immunity produced against citrate through a series of injections of hirudin. In one experiment, for instance, the blood of prepared mice coagulated in a citrate solution in 2 hours 2 minutes and in 1 hour 42 minutes, while the blood of control mice coagulated in the same solution in 2 hours and in 1 hour 29 minutes. In another experiment the blood of prepared mice coagulated in 2 hours 54 minutes while the blood of control mice coagulated in 1 hour 40 minutes and in 2 hours.

The serum of normal mice and of mice previously repeatedly injected with hirudin acts in approximately the same manner on the coagulation of a mixture of the blood of normal mice and citrate.

The serum of prepared mice added to a mixture of normal mouse blood and citrate caused coagulation in	21–24 hours (1 drop); 21–24 hours (2 drops); 7–15 hours (4 drops).
While the serum of control mice added to the same mixture caused coagulation in	21–24 hours (1 drop); 21–24 hours (2 drops); 1 hour 20 minutes (4 drops).

Action of oxalate on the blood of mice injected with hirudin.

14. Blood of normal mice and of mice immunized against hirudin coagulates in approximately the same time if received in a certain solution of oxalate of sodium.[7]

In solutions of oxalate of various strength the blood of prepared mice coagulated in	11 minutes in solution 1; 5 minutes in solution 2; 1 hour 6 minutes in solution 6.

[6] A solution of 0.33 per cent or of 0.25 per cent sodium citrate was usually used. Blood and solution were either mixed in equal amounts or the citrate solution was one-fourth of the mixture.

[7] Different dilutions of a 0.2 per cent stock solution of potassium oxalate were used.

While the blood of control mice coagulated in	10 minutes in solution 1; 4 minutes in solution 2; 1 hour 8 minutes in solution 6.

The serum of normal mice and of mice immunized against hirudin causes the coagulation of a mixture of normal mouse blood and oxalate in about the same time.

The serum of prepared mice caused the coagulation of a mixture of normal mouse blood and oxalate in	12 minutes (1 drop serum); 10 minutes (2 drops serum); 8 minutes (4 drops serum).
While the serum of normal mice caused the coagulation of the same mixture in	10 minutes (1 drop serum); 8 minutes (2 drops serum); 8 minutes (4 drops serum).
In a blood oxalate mixture, in which a stronger proportion of oxalate was present, prepared serum caused coagulation in	40 minutes (2 drops serum); 20 minutes (4 drops serum).
While control serum caused the coagulation of the same mixture in	40 minutes (2 drops serum); 23 minutes (4 drops serum).

Action of magnesium chloride on the blood of mice injected with hirudin.

15. Magnesium chloride acts in the same manner on the blood of prepared mice and of control mice.[8]

Serum of control mice causes coagulation of the blood of normal mice as rapidly as serum of prepared mice.

Blood of prepared mice collected in $MgCl_2$ solution coagulated in 29 minutes and 19 minutes, while the blood of control mice collected in $MgCl_2$ solution coagulated in 19 minutes; 20 minutes; 25 minutes; and 19 minutes.

In another experiment in which the proportion of $MgCl_2$ was greater, the blood of prepared mice coagulated in 10–14 hours in both samples, and the blood of control mice coagulated in 10–14 hours and in 16 hours, respectively.

[8] A 0.2–0.12 per cent solution of magnesium chloride was used.

The serum of prepared mice added to a mixture of blood and $MgCl_2$ caused coagulation in	16 minutes (1 drop serum); 13 minutes (2 drops serum); 7 minutes (4 drops serum).
While the serum of control mice caused coagulation in	15 minutes (1 drop serum); 13 minutes (2 drops serum); 6 minutes (4 drops serum).

We see, therefore, that immunization against hirudin does not produce immunity against the action of substances which prevent the clotting of the blood principally through inactivation of calcium, while immunization against hirudin produces also immunity against the anticoagulating effect of sodium fluoride.

The combined action of serum and tissue extract.

16. If we combine immune serum with tissue extract with or without calcium and allow the mixture to stand one minute before being added to hirudin normal mouse or rabbit blood, its action on hirudin blood is somewhat better than the action of immune serum alone. But under similar conditions the action of control serum is also improved.

In one experiment, for instance:

2 drops serum of prepared mice caused coagulation of hirudin mouse blood in 37 minutes; 2 drops of control serum caused coagulation in 5–15 hours, while 2 drops prepared serum plus 1 drop kidney extract caused coagulation in 13 minutes, and 2 drops control serum and 1 drop extract in 8–16 hours. In this experiment the beneficial action of the addition of extract to control serum is not shown.

17. Heating mouse serum to 56°C. diminishes its effect on fluoride blood very much, and the same holds good if the heated serum is combined with extract or with extract and calcium; this combination is much less active than fresh mouse serum and kidney extract with or without calcium.

Addition of kidney extract or of calcium and kidney extract to immune serum does not increase the activity of immune serum more than the addition of extract and calcium to control serum. If we mix 1 part extract and 1 part $CaCl_2$ with 2 parts mouse immune serum, the coagulation of fluoride mouse blood takes place in 21 hours; if we mix it with 2 parts control serum the coagulation

takes place in 30 hours. The same mixture with immune serum caused coagulation of rabbit fluoride-blood in 9–17 hours, and of mouse blood in 41–45 hours and in 21–30 hours, respectively.

We see, therefore, that addition of extract and calcium to immune serum did not in this experiment increase the activity of the latter very markedly. It does not increase the activity of immune serum more than of normal serum.

18. Through heating to 56° normal serum loses somewhat in its effect on citrate blood; it loses still more through heating to 60°. This applies to citrate blood in which the addition of a very small amount of calcium suffices to cause coagulation within a short time.

If we add extract or extract and calcium to control serum it behaves in a similar manner towards citrate blood as if we add the same substances to immune serum. These substances do not increase the efficacy of immune serum to a markedly greater extent than they do that of control serum. For instance, 2 parts immune serum and 1 part extract causes coagulation of citrate blood in 21 hours, while 2 parts control serum and 1 part extract causes coagulation of citrate blood in 24 hours.

19. The action of normal serum on $MgCl_2$ as well as on oxalate blood is weakened if the serum is heated to 56°C., and still more so if it is heated to 60°C. Extract may be just as active as serum or it may be more so in the case of $MgCl_2$ blood. It depends on the strength of the $MgCl_2$ solution used. The coagulation of $MgCl_2$ blood as well as of oxalate blood is very rapid after the addition of calcium, more rapid than with serum.

Addition of tissue extract to serum may accelerate both the action of immune and of normal serum on magnesium chloride blood, in accordance with the fact that extract alone may have a coagulating effect on the magnesium chloride blood, but the accelerating effect of the addition of extract to immune serum is not greater than the accelerating effect of the addition of extract to normal (control) serum. There is, therefore, no reason for assuming that there exists in the serum of the prepared animals an increased amount of the hypothetical thrombogen which is activated by the extract.

In one experiment two drops of immune serum caused coagulation of $MgCl_2$ rabbit blood in 41–46 hours; 2 drops of control

serum caused coagulation of the same $MgCl_2$ blood in 9–17 hours. A mixture of 1 part of immune serum and 2 parts extract caused coagulation in 41–46 hours; of 2 parts immune serum and 1 part extract in 3 hours 45 minutes, while the corresponding mixtures with control instead of immune serum caused coagulation in 7–8 hours and in 9 minutes, respectively.

20. If we compare the action of control serum, of calcium, of combinations of calcium and kidney extract and of combinations of control serum and kidney extract on the blood of prepared mice received in NaF, in $MgCl_2$ and citrate solutions and on blood of control mice received in similar solutions, we find that in each case these substances or combinations of substances act on both kinds of blood in a parallel way. There was no marked difference in the coagulation time of the control and prepared blood under the influence of the various substances; but the immune NaF blood coagulated in most cases somewhat more rapidly than the control NaF blood, just as the immune NaF blood alone coagulates usually more rapidly than the control NaF blood.

SUMMARY AND CONCLUSIONS.

1. If we give to mice daily intravenous injections of hirudin, a substance becomes demonstrable in the blood which antagonizes the anticoagulating effect of hirudin. This substance is active as well within the body of the injected animal as *in vitro;* in the whole blood as well as in the serum of the shed blood. As early as 4–5 days after the beginning of the injections, and 4–8 hours after the last immunizing injection, the presence of this substance can be demonstrated. 8–9 days after the last immunizing injection the presence of the immune substance is no longer demonstrable with the same regularity as at an earlier date. In some experiments in which a weaker hirudin was used, the acquired immunity was weak 24 hours after, and became more marked 2–3 days after the last injection.

2. The presence of a carcinoma in the mouse during the process of immunization seems to exert an inhibiting effect on the rapid appearance of the immune substance. Here also the immunity was in some cases more marked three days after, than one day after the last immunizing injection.

3. Heating the immune serum to 56° for 35 minutes did not decrease its coagulating power; heating it to 60° for 35 minutes decreased its power only slightly; while heating it to 65° caused its complete disappearance. This substance is therefore not identical with the thrombin of normal serum which is much more thermolabile.

4. The immune substance of the injected substance acts also on the hirudin blood of other species. If a relative species specificity exists at all—which is doubtful—it is certainly very slight.

5. The effect of the immune substance in counteracting the effect of hirudin is almost instantaneous. It is therefore probable that it acts by forming a combination with a substance inhibiting the coagulation of the blood and not through a fermentative or other change that it produces in this substance.

6. This immune substance antagonizes not only the anticoagulating action of proteid substances like hirudin and histon, but also of sodium fluoride, while it does not antagonize the anticoagulating effect of those substances that act through inactivation of calcium in the blood.

7. If we compare the coagulation of prepared and of control blood, the effect of the immunization is as marked in fluoride as in hirudin in some of the experiments; in others, however, it is more marked in hirudin. If we test the effect of the immunization on the coagulation of sodium fluoride blood with immune and normal serum, the effect of the immunization against hirudin is very much less marked than if we receive immune and control blood in sodium fluoride solution. On the whole, therefore, although the immunization against hirudin entails also a distinct immunization against sodium fluoride, the latter is not so marked as the immunity against hirudin.

8. The addition of tissue extract with or without calcium does not increase the effect of immune serum more than that of control serum, if both the combinations are tested in their effect on hirudin or sodium fluoride blood. Likewise addition of tissue extract to normal serum increases the activity of the latter in a similar manner, if the mixture is added to either normal or immune (against hirudin) fluoride blood. Also in the case of citrate and magnesium chloride blood the addition of tissue extract with (in the case of citrate blood) or without calcium does not increase the efficacy

of immune serum to a greater extent than it does increase the activity of control serum. In a similar manner, calcium, calcium plus tissue extract, serum plus tissue extract and control serum act in relatively the same way if the efficacy of these substances towards immune and control blood each received in a citrate and magnesium chloride solution is tested. We may, therefore, conclude that the immune substance found in the blood of animals treated with injections of hirudin is not identical with the hypothetical thrombogen.

9. We may, furthermore, conclude that the immune substance found in the blood of mice which received several intravenous injections of hirudin is not a direct antibody against the substance introduced; neither is it thrombin or thrombogen. In a provisional way we may at present consider the following two explanations:

(*a*) On entering the circulation hirudin combines with prothrombin. Various other substances inhibiting the coagulation of the blood, and among them sodium fluoride, are combined in some way with the same substance when mixed with the blood (anticoagulating substances of the first class). The substances, however, preventing the coagulation of the blood through inactivation of calcium (anticoagulating substances of the second class) do not combine with prothrombin. Through repeated injection of hirudin an antibody is produced against the combination of prothrombin-hirudin. This antibody, when mixed with the fibrinogen and prothrombin-hirudin (or histon or sodium fluoride) mixture, combines with the hirudin (or histon or sodium fluoride), inactivates this latter substance, therefore, to a great extent and sets the prothrombin partly or all free and the coagulation can now proceed. If, on the other hand, we introduce a substance inactivating merely all or a part of the calcium, the presence of this newly developed substance is of no consequence; neither is its presence of importance in the normal blood.

(*b*) In the circulating blood there exists an anticoagulating substance in combination with another protein substance. The injection of an anticoagulating substance of the first class frees the anticoagulating substance of the blood by entering in combination with the proteid with which this anticoagulating substance is normally combined. The anticoagulating substance of the

hlood being in an uncombined state acts like a foreign substance and calls forth the production of an antibody. If we mix this antihody with the hlood in which through the addition of the anticoagulating substance of the first kind the natural substance of the blood has been set free, the antibody combines with the latter entirely or in part, and the coagulation can proceed under the influence of the thrombin or tissue extract. Anticoagulating suhstances of the second class do not inhihit the coagulation of the blood by setting free the normal anticoagulating substance of the blood, but by inactivating calcium. In their case, therefore, as well as in the case of normal blood the presence of this antibody is irrelevant. We consider these explanations as of an entirely tentative character.

THE VALUE OF THE PROTEINS OF THE CEREAL GRAINS AND OF MILK FOR GROWTH IN THE PIG, AND THE INFLUENCE OF THE PLANE OF PROTEIN INTAKE ON GROWTH.[1]

By E. V. McCOLLUM.

(*From the Laboratory of Agricultural Chemistry of the University of Wisconsin.*)

(Received for publication, September 9, 1914.)

During recent years much interest has centered in the accumulation of quantitative data showing the relative amounts of the amino-acids yielded by different proteins after hydrolysis. The enthusiasm in this work is the result of the belief, now widely accepted, that the similarity in the composition of the food proteins, with respect to their yields of amino-acids, and those of the body which they are to replace or form anew, determines the relative values of the individual proteins as animal foods. That wide differences in the chemical nature of the proteins exist, has been clearly proven by the work of Fischer, Abderhalden, Kossel, Osborne and others. It has, however, been very difficult to produce definite experimental proof that comparable differences in the nutritive values of the individual proteins also exist. This involved the attainment of growth in young animals fed on rations made up of purified foodstuffs. In 1909 I published the first fairly successful experiments of this nature, and since that time a nearly complete solution of the problem of feeding such mixtures has been attained.[2] This work has been greatly extended by the excellent studies of Osborne and Mendel[3] who have compared

[1] Published with the permission of the Director of the Wisconsin Experiment Station.

[2] McCollum: *Amer. Journ. of Physiol.*, xxv, pp. 120–141, 1909; xxix, pp. 215–237, 1911; McCollum and Davis: this *Journal*, xiv, p. xl, 1913; xv, p. 167, 1913; also *Proc. Soc. Exp. Biol. and Med.*, xi, p. 101, 1914.

[3] Osborne and Mendel: *Publications of the Carnegie Institution of Washington, Bulletin 156*, Parts 1 and 2, 1911 and 1912; also this *Journal*, xii, pp. 81–89, 1912; xiii, p. 233, 1912; xv, pp. 311–326, 1913; xvi, pp. 423–427 1913.

numerous individual proteins as sole sources of nitrogen in the diets of rats. The investigations referred to have now established the fact that certain individual proteins from both animal and vegetable sources are capable of supplying everything necessary in the way of nitrogen-containing complexes for prolonged growth. Other proteins, as gliadin of wheat, suffice for maintenance, but not for growth; while still others, as gelatin or zein, can serve only in part to replace the nitrogen lost through endogenous metabolism and are incapable, when fed singly, of inducing growth in young animals.

While such studies relating to the values of isolated proteins are of fundamental importance in revealing the character of the chemical processes involved in nutrition, practical dietetics and animal production must always deal with certain groups of proteins as they are found in the naturally occurring foodstuffs. In the maize kernel zein is present to the extent of about half of the total protein content of the grain. This protein has been shown by Willcock and Hopkins[4] to be insufficient for prolonged maintenance of mice. McCollum[5] in experiments covering more than thirty days observed persistent negative nitrogen balances, but pigs appear to utilize as much as 80 per cent of the nitrogen of zein for maintenance, but none for growth. Osborne and Mendel[6] have observed a steady decline in rats taking zein as the sole protein in the diet.

Quantitative data are wanting to show to what extent the remaining proteins of the maize kernel supplement the deficiencies of zein in producing a mixture of protein cleavage products which is suitable for the construction of body proteins during growth. Hart, Humphrey and Morrison[7] have, however, shown that the corn grain proteins, supplemented with a small proportion of the proteins of corn fodder, served for growth with calves, with a storage of approximately 20 per cent of the ingested nitrogen. Osborne and Mendel[8] have recently shown that maize glutelin

[4] Willcock and Hopkins: *Journ. of Physiol.*, xxxv, p. 88, 1906.
[5] McCollum: *Amer. Journ. of Physiol.*, xxix, p. 215, 1911.
[6] Osborne and Mendel: this *Journal*, xvii, p. 345, 1914.
[7] Hart, Humphrey and Morrison: this *Journal*, xiii, p. 133, 1912.
[8] Osborne and Mendel: this *Journal*, xviii, p. 12, 1914.

serves as an adequate source of protein for growth during ninety days in the rat.

The present paper is the second report of a series of experiments which have been in progress in this laboratory during the last four years, with a view to obtaining quantitative data on the relative values of the protein mixtures occurring in the natural foodstuffs. One cannot at present give a definite answer to the question whether a given mixture of protein cleavage products is equally well suited for quantitative rearrangement into the specific body proteins of one species of animal as another. The anaphylactic reaction gives definite evidence that the tissue proteins of one species are foreign proteins to another species, but we know nothing as to the nature of these differences, whether they involve appreciable differences in the percentages of the various amino-acids, or rest in a different mode of arrangement of these complexes. If the latter were the case we should expect the nutritive value of any protein to be as great for one species as for another.

A question of fundamental importance, in a study of this kind, where it is sought to find the maximum possible efficiency with which an animal can transform the proteins of a given foodstuff into body protein during growth, is the selection of that species which has the greatest capacity to grow, but more especially the greatest growth impetus. Very slow growing species, as man, cannot be expected to store the nitrogen of the food during growth to the maximum extent possible with the particular food proteins consumed. In the child the growth impetus is low, the initial weight at birth being multiplied by three during the first year of life. A rat at birth weighs on an average 4.83 grams and contains on an average 0.064 gram of nitrogen. At 280 grams' body weight the nitrogen content of a fairly fat rat is 8.5 grams. During a period of 280 days, which is the normal growing period for this species, the animal multiplies its initial nitrogen content by 133. During the same interval the initial body weight is multiplied about fifty-five times.

The new-born pig of two pounds' weight contains 134 grams of dry matter and 11.9 grams of nitrogen. In 280 days, with a good diet, it will reach a body weight of 300 pounds. The nitrogen

content of an average moderately fat hog of this size is at least 2407 grams. This indicates a multiplication of the initial nitrogen content by 202.[9]

It is evident that the growth impetus is greater in the pig than in the rat, and it is doubtful whether the pig is exceeded in this respect by any other animal. The results obtained with this species will, therefore, more closely approximate the values for the maximum possible efficiency with which the animal body can convert any mixture of amino-acids derived from a single protein or a mixture of proteins in a naturally occurring foodstuff into new body proteins than can be obtained with any other species, unless the data with other species represent the performance of the body during the first days of postnatal life.

Another question which arises in the study of this problem is the effect of the plane of protein intake on the rate of nitrogen retention. Animal husbandry experience has long ago established the fact that young animals do not grow well on a low protein diet, and the practice of feeding rations containing from 20 to 25 per cent of protein to young pigs and calves is regularly taught in the agricultural colleges at the present time. On teleological grounds we should assume that the content of protein carried by milk should represent the optimum proportion of this constituent to the remaining portion of the diet. The milk of the cow, free from water, contains more than 30 per cent of protein, that of the sow 35 per cent, according to König. Osborne and Mendel[10] have published results which indicate that rats do not grow unless the ration contains more than 12 per cent and less than 30 per cent of protein. Their data indicate that rats lose weight steadily when fed a ration containing 32 per cent of protein. In a former notice McCollum and Davis[11] have called attention to the fact that this is by no means necessarily the case. Rats in our colony have grown well during more than two months on rations containing as low as 12 per cent and as much as 34 per cent of protein.

[9] These figures are from unpublished data obtained in this laboratory.

[10] Osborne and Mendel: *Zeitschr. f. physiol. Chem.*, lxxx, p. 307, 1912.

[11] McCollum and Davis: *Proceedings Amer. Soc. Biol. Chem.*, this *Journal*, xiv, p. xi, 1913.

PLAN OF THE EXPERIMENTS.

Thrifty young pigs from the University stock were placed in metabolism cages and fed starch, agar-agar and water until the feces failed to show evidence of being derived from the grain previously fed. A ten-day period of quantitative collection of the feces and urine then followed on the starch diet. The nitrogen of the feces was determined and the total nitrogen and the creatinine were determined in the urine. The diet was then changed from starch to the grain in question with or without the addition of starch, or a protein concentrate, as described in the tables. Total nitrogen was determined in the urine and feces. In most of the experiments the grain feeding was continued for forty to sixty days, then the ration was again changed to starch, agar-agar and water for a period of ten days during which total nitrogen of the feces and urine and the creatinine excretion were determined.

In order to have a standard for the amount of protein to be introduced into the diets so that as nearly as possible comparable amounts of protein should be fed to different animals of the series, the protein intake was based upon the creatinine output of the animal during the preliminary starch period. It was assumed that the endogenous upkeep of the pig required 5.5 times the nitrogen daily eliminated as creatinine,[12] since on long continued starch feeding the creatinine nitrogen has been observed to form 18.5 per cent of the total urinary nitrogen. This plan certainly approximates much more closely a comparable adjustment of the protein of the rations of a series of animals to the amount of metabolizing tissue than can be attained by basing the calculation of the ration on the body weight.

The energy content of the rations was in most of the experiments kept very near 100 calories per kilo per day.

In expressing the results the per cent of the ingested nitrogen and the per cent of absorbed nitrogen have both been calculated. There is no doubt that a certain portion of the nitrogen of the feces does not represent undigested food residues, but unabsorbed intestinal secretions, cast off cells, bacteria, etc. The origin of this portion of the fecal nitrogen has been discussed at length by

[12] McCollum: *Amer. Journ. of Physiol.*, xxix, p. 210, 1911.

a number of investigators and need not be considered here.[13] It should be taken into account in judging as to the per cent of the food nitrogen which is converted into body tissue. In arriving at the value for "per cent of absorbed N retained" that amount of nitrogen is deducted from the total fecal nitrogen, which it was estimated would have appeared in the feces during the entire feeding period if the animal had been kept on the nitrogen-free diet of starch and agar-agar. The value for this fraction was based on the results for the preliminary starch period. The remainder of the data in the tables will be readily interpreted.

DISCUSSION OF RESULTS.

Table I gives summaries of the data obtained in the feeding experiments discussed in this paper. Certain data for all experiments are summarized in Table II for ease of comparison.

1. With moderately low protein intake (6.6–10 per cent) the rate of nitrogen retention is influenced by the amount of food protein in proportion to the metabolizing tissues of the body, and apparently in some degree by the excess of total energy consumed over the maintenance needs of the animal.

2. When the energy supply is generous (100 cal. per kgm. or more) the rate of nitrogen retention as expressed in per cent of ingested nitrogen is not much influenced by the plane of protein intake at levels above 10 per cent of the ration. In experiments in which the protein amounted to 41 to 57.86 per cent of the ration the maximum possible per cent of the ingested nitrogen continues to be retained for growth.

The results all point to the belief that in the young pig the growth impulse is so great that the synthesis of body protein is effected at the maximum rate possible with the particular mixture of amino-acids yielded by the food proteins. If this be true the figures for the per cent of the absorbed nitrogen retained for new growth indicate the degree to which the amino-acids of the food can be recombined into tissue proteins and they represent comparative numerical values for proteins from different sources.

[13] See Tsuboi: *Zeitschr. f. Biol.*, xxxv, p. 68, 1897; McNeal, Latzer, and Kerr: *Journ. of Infect. Diseases*, vi, p. 123, 1909; Mendel and Fine: this *Journal*, xi, p. 5, 1912.

While there is some variation among the experiments here reported, the data show that there is very little difference in the value for growth of the protein mixtures contained in the three cereal grains, wheat, oat and corn kernels. A maximum of 23–24 per cent of the ingested nitrogen from one of these sources can be retained for growth.

In a single experiment in which the three grains were fed together in equivalent amounts there did not appear to be a very appreciable favorable influence on the nitrogen retention due to the supplementing action of the proteins from one source on those from another. No conclusion can be drawn from one experiment and it is easily conceivable that mixtures of two or more of the cereal grains in certain proportions may supply the amino-acids necessary for growth in very favorable proportions. Further data on this point will be collected.

The experiments in feeding at very high planes protein intake deserve a few words of comment. It has not been found possible to secure a very high intake of protein by feeding corn meal and corn gluten, and since no commercial protein concentrate for the oat kernel is available no effort has yet been made to feed high protein rations derived from the latter.

Wheat embryo proved sufficiently appetizing to induce pigs to consume a liberal energy intake of mixtures of wheat embryo and wheat gluten. Since the protein content of the latter is about 75 per cent, almost any desired protein content in the ration could be secured. In the three experiments with such mixtures (Exps. 17, 18, 19) the protein contents of the rations were 57.86, 54.22 and 41.38 per cent, and the energy intake 98.0, 107.5 and 96.5 calories per kilo respectively. The protein furnished by the wheat embryo was 29.7, 34.14 and 31.08 per cent of the total. The per cent of ingested nitrogen retained in the three experiments was 21.39, 19.76 and 25.88 per cent respectively. One cannot determine from the data at hand whether the different proportions between the embryo proteins and the endosperm proteins of the gluten are responsible for the variation in the rate of retention. The figures are of the same order as those obtained for the wheat kernel alone and for the mixtures of the wheat kernel and wheat gluten, and altogether the results would seem to leave little doubt *that the rate of retention of nitrogen, in all cases*

TABLE I.

Wt. of Pig		Creatinine N output		N from endogenous metabolism	Fed per day		Calories per day (estimated)		Per cent of protein in ration (N × 6.25)	N in ration	N in urine	N in feces			Total N excreted	N absorbed	N retained	Per cent of N intake absorbed	Per cent of N intake retained	Per cent of absorbed N retained
Initial	Final	Initial	Final		N	Protein equivalent	Total	Per kilo				Total	From food	Not from food						
kilos	kilos	grams	grams	grams	grams	grams				grams	grams	grams	grams	grams	grams	grams	grams			
Exp. 1. Pig No. 15. Fed 5 × Maintenance (oats). Composition of Ration: Rolled oats, 274 grams; starch, 336 grams. (Feeding period 55 days.)																				
24.6	30.7	0.2683	0.2871	1.4795	7.39	46.19	2460	100	7.22	406.5	266.55	97.05	56.9	40.15	363.60	349.6	42.9	86.02	10.55	12.27
Exp. 2. Pig No. 16. Fed 5 × Maintenance (wheat). Composition of Ration: Wheat, 489 grams; starch, 288 grams. (Feeding period 56 days.)																				
23.4	32.5	0.3022	0.2914	1.6620	8.31	51.94	2840	100	6.68	465.4	320.98	95.88	47.72	48.16	416.86	417.7	48.6	89.9	10.44	11.51
Exp. 3. Pig No. 26. Fed 5 × Maintenance (corn). Composition of Ration: Corn meal, 484 grams; starch, 90 grams. (Feeding period 45.5 days.)																				
20.9	23.6	0.2802	0.2708	1.5411	7.93	49.56	2090	100	8.63	361.0	224.22	76.11	43.35	32.76	300.33	317.65	60.67	87.98	16.80	19.09
Exp. 4. Pig No. 27. Fed 7 × Maintenance (oats). Composition of Ration: Rolled oats, 270 grams; starch, 109 grams. (Feeding period 24 days.)																				
15.0	18.4	0.1893	0.1943	1.0411	7.29	45.56	1500	100	12.02	178.5	132.73	22.03	9.79	12.24	154.74	168.71	23.8	94.51	13.33	14.11
Exp. 5. Pig No. 29. Fed 7 × Maintenance (wheat). Composition of Ration: Wheat, 566 grams. (Feeding period 50 days.)																				
21.9	26.4	0.2786	0.3015	1.5300	10.72	67.00	2066	94	11.82	536.0	353.6	90.1	50.1	40.0	443.7	485.9	93.0	90.65	17.35	19.14
Exp. 6. Pig No. 30. Fed 7.5 × Maintenance (corn). Composition of Ration: Corn meal, 455 grams. (Feeding period 50 days.)																				
20.0	21.4	0.1837	0.2142	1.0100	7.65	47.81	1651	82.5	10.5	382.5	239.8	82.3	48.3	34.0	322.1	334.2	60.43	87.37	15.80	18.08
Exp. 7. Pig No. 24. Fed 10 × Maintenance (corn). Composition of Ration: Corn meal, 825 grams. (Feeding period 53 days.)																				
27.5	33.2	0.2519	0.2998	1.3854	14.0	87.5	2995	108.8	10.5	741.0	374.0	206.0	168.4	37.6	580.0	572.6	161.0	77.28	20.92	28.12
Exp. 8. Pig No. 21. Fed 10 × Maintenance (corn). Composition of Ration: Corn meal, 638 grams. (Feeding period 50 days.)																				
17.3	20.0	0.2142	0.2722	1.0710	10.71	66.94	2316	133.8	10.49	535.5	263.7	148.2	118.2	30.0	411.9	417.3	123.6	77.96	23.08	29.60
Exp. 9. Pig No. 14. Fed 10 × Maintenance (wheat). Composition of Ration: Wheat, 726 grams. (Feeding period 62 days.)																				
25.7	30.9	0.2323	0.3070	1.2776	12.74	79.62	2650	103	11.0	802.3	453.7	174.5	132.5	42.0	62.81	669.8	174.2	83.48	21.67	26.01
Exp. 10. Pig No. 13. Fed 10 × Maintenance (oats). Composition of Ration: Rolled oats, 498 grams. (Feeding period 60.5 days.)																				
21.8	29.0	0.2168	0.2870	1.1590	11.62	72.62	2029	93.7	14.54	703.0	416.5	134.8	92.8	42.0	542.3	610.2	161.0	86.65	22.9	26.38
Exp. 11. Pig No. 25. Fed 10 × Maintenance (corn meal ½, wheat ¼, rolled oats ¼). Composition of Ration: Starch, 273 grams; corn meal, rolled oats, wheat, 140 grams each. (Feeding period 45.5 days.)																				
26.36	30.5	0.2962	0.3863	1.6290	16.48	103.0	2589	98.2	12.26	749.8	380.3	172.0	143.34	28.66	552.3	606.46	197.5	80.87	26.34	32.56

Exp. 12. Pig No. 18. Fed 15 × Maintenance (corn). Composition of Ration: Corn meal, 775 grams. (Feeding period 62.5 days.)

19.8	23.7	0.1580	0.2020	0.8690	13.03	81.43	2813	142.0	10.5	814.37	374.76	228.43	187.81	40.62	603.19	626.56	211.17	76.93	25.93	33.70

Exp. 13. Pig No. 19. Fed 15 × Maintenance (wheat). Composition of Ration: Wheat, 598 grams. (Feeding period 23 days.)

12.9	15.5	0.1079	0.1260	0.5930	8.89	55.56	1850	143.0	10.93	204.7	104.0	53.94	42.9	11.01	157.94	161.8	46.79	79.01	22.86	28.92

Exp. 14. Pig No. 22. Fed 15 × Maintenance (wheat). Composition of Ration: Wheat, 600 grams; wheat gluten, 70 grams. (Feeding period 60 days.)

24.32	31.13	0.2280	0.2380	1.2540	19.0	118.75	2470	101.0	17.72	1140.0	712.6	172.8	124.2	48.6	885.4	1015.8	254.6	89.10	22.33	25.06

Exp. 15. Pig No. 23. Fed 20 × Maintenance (corn). Composition of Ration: Corn meal, 400 grams; corn gluten, 188 grams. (Feeding period 46 days.)

17.7	20.7	0.2052	0.2175	1.1200	22.6	141.2	2204	124.0	24.02	1039.0	549.2	202.8	179.3	23.46	752.0	859.7	257.0	82.74	24.73	29.89

Exp. 16. Pig No. 20. Fed 15 × Maintenance (oats). Composition of Ration: Rolled oats, 474 grams. (Feeding period 60 days.)

16.4	23.2	0.1552	0.2374	0.8536	12.8	80.0	1896	115.0	16.87	640.0	384.16	78.30	52.8	25.5	462.46	587.2	177.66	91.75	27.76	30.25

*Exp. 17. Pig No. 29. High Protein Feeding. Composition of Ration: Wheat embryo, 320; wheat gluten, 300 grams. (29.7 per cent of protein from wheat embryo; 70.3 per cent from wheat gluten.) (Feeding period 39.5 days.)**

26.4	30.7	0.3015	0.5369	1.6582	57.4	358.75	2609	98.0	57.86	2267.3	1665.97	116.5	84.1	32.39	1782.47	2183.2	484.9	96.24	21.39	22.21

Exp. 18. Pig No. 36. High Protein Feeding. Composition of Ration: Wheat embryo, 159 grams; wheat gluten, 141.5 grams. (34.14 per cent of protein from wheat embryo, 65.86 per cent from wheat gluten.) (Feeding period 50 days.)

11.8	21.37	0.1083	0.1188	0.5956	26.07	162.9	1268	107.5	54.22	1303.47	980.68	65.2	44.2	21.0	1045.88	1259.27	257.59	96.69	19.76	20.45

Exp. 19. Pig No. 35. High Protein Feeding. Composition of Ration: Wheat embryo, 114.3 grams; wheat gluten, 107 grams. (31.08 per cent of protein from wheat embryo; 68.92 per cent from wheat gluten.) (Feeding period 26 days.)

10.8	12.7	0.0981	1.1452	0.5395	19.07	119.2	965.0	96.5	41.38	505.35	344.18	30.38	19.2	11.18	374.56	486.15	130.79	96.20	25.88	26.00

Exp. 20. Pig No. 42. Fed 10 × Maintenance (casein + starch + salts). Composition of Ration: Casein, 79.4 grams; starch, 400 grams; agar-agar, 10 grams; salt mixture, 25.77 grams.† (Feeding period 33 days.)

17.0	23.2	0.1536	0.2880	0.8445	10.0	65.28	1600	94.1	15.5	520.0	141.0	50.0	23.0	27.04	191.0	497	329	95.57	63.27	66.19

Exp. 21. Pig No. 8. Fed 11.9 × Maintenance (skim milk and starch). Composition of Ration: milk, 2.1 liters; starch, 200 grams. (Feeding period 52 days.)

17.3	22.0	0.1738	0.2448	0.9559	10.0	65.25	1788	102.0	16.5	330.0	128.4	48.9	33.4	15.51	177.3	296.6	150.2	89.88	45.51	50.64

*After 39.5 days the pig refused to eat the ration and was at once fed starch only. It is evident that such a high protein intake is injurious if long continued.

†Composition of salt mixture: NaCl, 2.457; $MgSO_4$ (anhydrous), 1.160; $Na_2SO_4 + 7\,H_2O$, 2.153; K_2SO_4, 0.822; KH_2PO_4, 3.876; $K_3C_6H_5O_7 + H_2O$ (K citrate), 3.833; $Ca(C_3H_5O_3)_2 + 5\,H_2O$ (Ca lactate), 11.475 grams. Total, 25.776 grams.

TABLE II.

Summary of results of feeding proteins from various sources and at different planes.

SOURCE OF PROTEIN	MULTIPLE OF MAINTENANCE REQUIREMENT FED (N)	PER CENT OF PROTEIN IN RATION	CALORIES PER KGM.	PER CENT OF INGESTED N RETAINED FOR GROWTH	PER CENT OF ABSORBED N RETAINED FOR GROWTH	EXP. NO. (SEE TABLE I)
Oats	5.0	7.22	100.0	10.55	12.27	1
Wheat	5.0	6.68	100.0	10.44	11.51	2
Corn	5.0	6.63	100.0	16.80	19.09	3
Oats	7.0	12.02	100.0	13.33	14.11	4
Wheat	7.0	11.82	94.0	17.35	19.14	5
Corn	7.5	10.50	82.5	15.80	18.08	6
Corn	10.0	10.50	108.8	20.92	28.12	7
Corn	10.0	10.50	133.8	23.08	29.60	8
Wheat	10.0	11.00	103.1	21.67	26.01	9
Oats	10.0	14.54	93.7	22.90	26.38	10
Oats ⅓, Wheat ⅓, Corn ⅓	15.0	12.26	98.2	26.34	32.56	11
Corn	15.0	10.50	142.0	25.93	33.70	12
Wheat	15.0	10.93	143.0	22.86	28.92	13
Wheat	15.0	17.72	101.0	22.33	25.06	14
Corn	20.0	24.20	124.0	24.73	29.89	15
Oats	15.0	16.87	115.0	27.76	30.25	16
Wheat embryo and wheat gluten	34.8	57.86	98.0	21.39	22.21	17
Same	43.9	54.22	107.5	19.76	20.45	18
Same	35.4	41.38	96.5	25.88	26.90	19
Casein	10.0	16.50	102.0	45.51	50.64	20
Skim milk	11.9	15.50	94.1	63.27	66.19	21

where a sufficiently high plane of protein intake was fed, was limited by the chemical make-up of the food proteins, and not by the physiological capacity of the animals to grow.

In support of this statement I submit the results of an experiment in which the food consisted of casein and starch with a salt mixture, Exp. 21, and one in which skim milk and starch were fed, Exp. 20. Of the ingested nitrogen 45.51 was retained when casein was the sole source of protein, and 63.27 per cent when skim milk was given. The physiological limit of growth capacity was not attained in the experiments in which all protein was

derived from cereal grains. There is no room for doubt that the protein mixture occurring in either the wheat, oat or corn kernel is chemically inferior to casein alone or to the protein mixture afforded in milk. The data seem equally convincing that the protein mixture in each of these grains is, singly, adequate chemically for the complete formation of the specific proteins of the pig's body, although quantitatively the possibility for this conversion is relatively low.

There was always a significant rise in the amount of nitrogen eliminated as creatinine in all cases where a fairly large amount of nitrogen was retained for growth. This would appear to strengthen the evidence that all the necessary cleavage products of proteins necessary for the construction of metabolizing tissue in the animal were supplied by the proteins of each of the cereal grains employed.

In conclusion I would call attention to the fact that the data here reported throw no light on the value of the single grain rations for long continued growth and well-being. In numerous pen feeding experiments which have been carried out with such rations at this Station we have always observed that after two or three months signs of inadequate nutrition appear. The character of the mineral element supply furnished by rations restricted to a single grain, and the possible presence of small amounts of substances of harmful nature, or perhaps the lack of some, at present unappreciated, constituents necessary for prolonged physiological well-being, suggest themselves, as explanations. The experiments reported in this paper are of so short duration that these factors do not enter in appreciable degree. Mention should, however, be made that there was always evident toward the close of these feeding experiments a tendency for the rate of nitrogen retention to fall slightly, and the results of pen feeding over longer periods leave no doubt that a steady decline in the rate of growth ultimately sets in. In a forthcoming paper from this laboratory we will report the results of our efforts to determine what factors are responsible for failure of nutrition on rations restricted to the corn and wheat kernels.

THE EFFECT OF ALKALI ON PERMEABILITY.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University.)

(Received for publication, September 9, 1914.)

Warburg[1] has shown that NaOH greatly increases the oxidation of fertilized eggs of the sea urchin. He also found by staining the eggs with neutral red that the NaOH does not penetrate into the interior of the egg. Harvey has found that NaOH does not readily penetrate the cells of *Elodea*, *Spirogyra*, *Paramoecium* and eggs of various echinoderms.[2]

The very important question arises: How can NaOH affect oxidation when its action is confined to the surface of the cell? We might suppose that oxidation takes place principally at the surface; but it seems possible that NaOH affects oxidation by increasing the permeability of the egg for oxygen or for other substances.[3]

The writer has made experiments which show that the permeability of the protoplasm is increased by NaOH. The method employed was to make determinations of the electrical resistance of living tissues of *Laminaria saccharina* by a method which has been previously described.[4] Such determinations afford an accurate measure of the permeability of the protoplasm.

A solution of NaOH of the same conductivity as the sea water was prepared (about 0.22 M); 48 cc. of this solution were added to 1975 cc. of sea water, making the concentration of NaOH about 0.0052 M. A precipitate was formed which disappeared on

[1] *Zeitschr. f. physiol. Chem.*, lxvi, p. 305, 1910; *Biochem. Zeitschr.*, xxix, p. 414, 1910.

[2] Cf. *Amer. Journ. of Physiol.*, xxxi, p. 335, 1913, where references to earlier papers will be found.

[3] Increasing the permeability of the egg for products of oxidation would increase oxidation.

[4] *Science*, N. S., xxxv, p. 112, 1912.

shaking. The resistance was slightly increased so that 13 cc. of the sea water of double strength had to be added to restore the original resistance. The NaOH undergoes a reaction with the magnesium in the sea water forming magnesium hydrate. The actual alkalinity of the sea water therefore depends on the dissociation of the very small amount of magnesium hydrate which goes into solution. The sea water + NaOH was decidedly alkaline to phenolphthalein, to rosolic acid and to neutral red and slightly alkaline to litmus.

The addition of NaOH slightly decreases the number of magnesium ions, but this effect is too small to produce any change in permeability.

In sea water 1975 cc. + NaOH 0.22 M 48 cc. (= 0.0052 M) the resistance of the tissue steadily decreased. The results of a typical experiment are shown in Table 1 and Chart 1. During th first hour the fall amounted to 6 per cent;[5] in two and a half hours it fell 14 per cent, and at the end of five and five-sixths hours it had fallen 32 per cent.

It will be noticed that the resistance falls rapidly at first and then remains stationary for a short time. The writer hopes to throw some light on this by means of future experiments.

In order to test the effect of stronger concentrations of alkali, a mixture of 1793 cc. NaCl 0.52 M + 207 cc. $CaCl_2$ 0.279 M was prepared. (To this alkali may be added without causing precipitates such as would be formed in sea water.) A lot of tissue which had in sea water a resistance of 860 ohms (including the resistance of the apparatus) was placed in this mixture; in thirty minutes the net resistance increased to 117 per cent of the original net resistance; after one hour it was 119 per cent, after three hours 113 per cent, after six and two-thirds hours 83 per cent. The rise in resistance was due to the relatively large amount of $CaCl_2$ in the mixture. In the same mixture + 0.001 M NaOH the results were practically the same. With the addition of 0.01 M NaOH to the mixture the resistance was considerably lower. A lot of tissue which had in sea water a resistance of 940 ohms was placed in the mixture of NaCl + $CaCl_2$ and sufficient NaOH solution of the

[5] This is the per cent of the net resistance of the tissue in sea water. The net resistance is obtained by subtracting the resistance of the apparatus from the resistance as read.

TABLE 1.

TIME IN HOURS	ELECTRICAL RESISTANCE OF LAMINARIA SACCHARINA IN	
	Sea water 1975 cc. + NaOH 0.22 M 48 cc. (= 0.0052 M NaOH)	Sea water
	per cent	*per cent*
0	100	100
½	94	
1	94	
2½	86	
4⅙	74	
5⅚	68	100

All readings were taken at 18°C. The percentages were calculated on the basis of the net resistance in sea water at the beginning of the experiment.

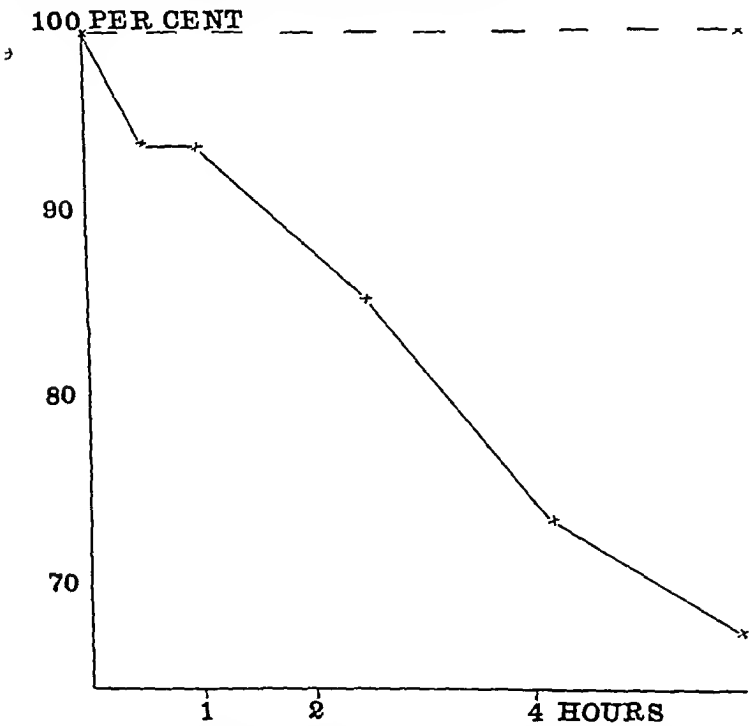


CHART 1. Curve of electrical resistance of *Laminaria saccharina* in 1975 cc. sea water + 48 cc. NaOH 0.22 M (=0.0052 M) (unbroken line) and of a control in sea water (dotted line). The percentages were calculated on the basis of the net resistance in sea water at the beginning of the experiment.

same conductivity as sea water was added to make the concentration of NaOH 0.01 M. After thirty minutes the net resistance rose to 109 per cent; after one hour it was 95 per cent; after three hours 70 per cent; and after six and two-thirds hours 39 per cent. On comparing this with the resistance of the control in NaCl and $CaCl_2$ without addition of alkali it will be seen that it is everywhere lower (Table 2 and Chart 2). The comparison is expressed graphically in Chart 3; the ordinates in this figure represent the net resistance as compared with that of the control (which is itself expressed as percentage of the original net resistance). Thus after thirty minutes the net resistance of the control had risen to 117 per cent, while that of the tissue in the mixture + 0.01 M

TABLE 2.

TIME IN HOURS	ELECTRICAL RESISTANCE OF LAMINARIA SACCHARINA IN (1793 cc. NaCl 0.52 M + 207 cc. $CaCl_2$ 0.279 M) CONTAINING				
	0 NaOH	0.001 M NaOH	0.01 M NaOH	0.02 M NaOH	0.03 M NaOH
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
0	100	100	100	100	100
½	117	117	109	108	106
1	119	118	95	91	85
3	113	112	70	53	43
6⅔	83	81	39	28	22

All readings were taken at 18°C. The percentages were calculated on the basis of the net resistance in sea water at the beginning of the experiment.

NaOH had risen only to 109 per cent; hence the rise was in this case only (109 ÷ 117 =) 93 per cent of that of the control; accordingly the ordinate erected at this point is 93. Proceeding in the same way we find that after one hour the rise was (95 ÷ 119 =) 80 per cent of that of the control; after three hours it was (70 ÷ 113 =) 62 per cent; after six and two-thirds hours it was (39 ÷ 83 =) 47 per cent. These results together with those obtained with stronger concentrations of NaOH (0.02 M and 0.03 M) are shown in Table 3 and Chart 3.

The data given in Table 3 are plotted in Chart 3A in such a manner as to show more clearly the effect of increasing concentrations of NaOH.

The addition of sufficient NaOH to make its concentration 0.02 M or 0.03 M must precipitate some of the Ca as $Ca(OH)_2$ since

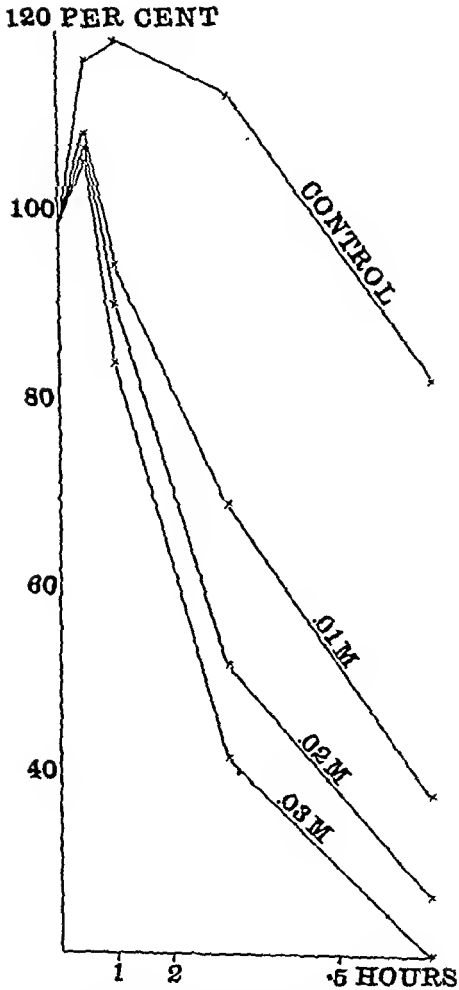


CHART 2. Curves of electrical resistance of *Laminaria saccharina* in (1793 cc. NaCl 0.52 M + 207 cc. $CaCl_2$ 0.279 M) with the addition of various amounts of NaOH. The percentages were calculated on the basis of the net resistance in sea water at the beginning of the experiment.

the latter is not soluble to the extent of 0.02 M. And even the addition of sufficient NaOH to make the concentration 0.01 M, though not resulting in the precipitation of $Ca(OH)_2$, must reduce

the number of calcium ions by the formation of $Ca(OH)_2$, which does not dissociate as much as $CaCl_2$.

A somewhat different method of procedure was adopted in another series of experiments. To 1000 cc. NaCl 0.52 M there was added 0.952 gram CaO; to this 100 cc. of distilled water was added to make the conductivity equal to that of sea water. The dissolved molecules therefore consisted of 97.2 per cent NaCl and 2.8 per cent $Ca(OH)_2$.

TABLE 3.

TIME IN HOURS	ELECTRICAL RESISTANCE OF LAMINARIA SACCHARINA IN (1793 cc. NaCl 0.52 M + 207 cc. $CaCl_2$ 0.279 M) CONTAINING				
	0 NaOH	0.001 M NaOH	0.01 M NaOH	0.02 M NaOH	0.03 M NaOH
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
0	100	100	100	100	100
½	100	100	93	92	91
1	100	100	80	76	71
3	100	100	62	47	38
6⅔	100	99	47	34	27

All readings were taken at 18°C. The percentages were calculated on the basis of the net resistance of the control.

For purposes of comparison another solution was prepared by mixing together NaCl 0.52 M and $CaCl_2$ 0.279 M (both of which have the same conductivity as sea water) in such proportions that the dissolved molecules consisted of 97.2 per cent NaCl and 2.8 per cent $CaCl_2$. Tissue was placed in each of these mixtures and also in NaCl 0.52 M. The results are shown in Table 4 and Chart 4. While the resistance fell very slowly in NaCl 97.2 per cent + $CaCl_2$ 2.8 per cent, it fell almost as rapidly in NaCl 97.2 per cent + $Ca(OH)_2$ 2.8 per cent as it did in NaCl. The concentration of $Ca(OH)_2$ was 0.015 M.

Since the addition of this amount of alkali causes some reduction in the concentration of calcium ions (though not in the concentration of calcium molecules) an experiment was made in which the concentration of calcium ions was kept undiminished. For this purpose there was added to a saturated solution of $Ca(OH)_2$ in water sufficient $CaCl_2$ 1.42 M to make the conductivity equal to that of sea water. Tissue was placed in this and also in $CaCl_2$ 0.278 M.

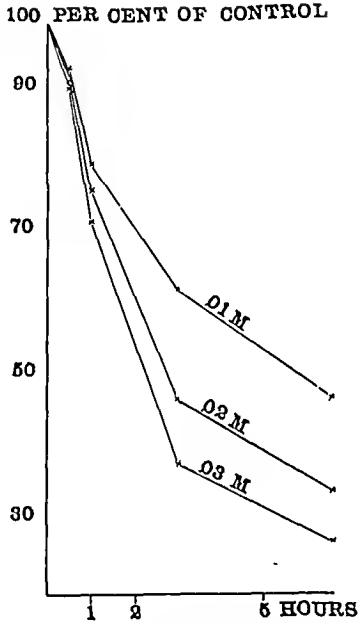


CHART 3.

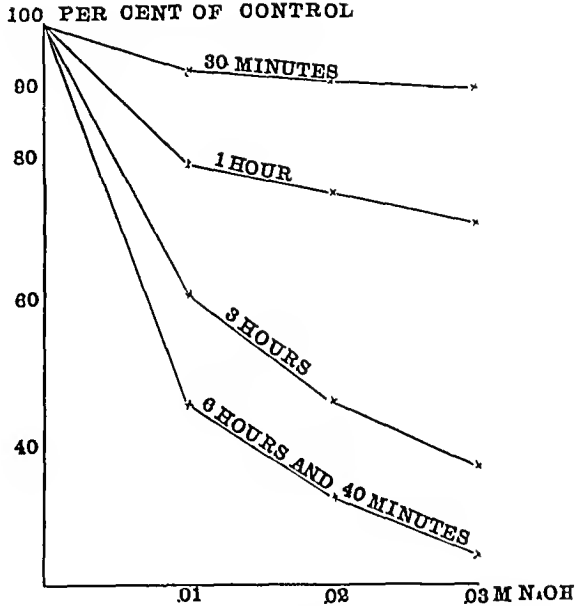


CHART 3A.

CHART 3. Curves of electrical resistance of *Laminaria saccharina* in (1793 cc. NaCl 0.52 M + 207 cc. $CaCl_2$ 0.279 M) with the addition of various amounts of NaOH. The percentages were calculated on the basis of the net resistance of the control.

CHART 3A. Curves of electrical resistance of *Laminaria saccharina* in (1793 cc. NaCl 0.52 M + 207 cc. 0.279 M) containing various amounts of NaOH. The abscissae represent the concentration of NaOH in the solution: the ordinates represent the percentage of electrical net resistance calculated on the basis of the net resistance of the control.

TABLE 4.

TIME IN HOURS	ELECTRICAL RESISTANCE OF LAMINARIA SACCHARINA IN		
	NaCl 97.2 per cent + $CaCl_2$ 2.8 per cent	NaCl 97.2 per cent + $Ca(OH)_2$ 2.8 per cent	NaCl
	per cent	*per cent*	*per cent*
0	100	100	100
½	98	87	73
1	97	73	56
2	94	45	37
3½	92	34	27

All readings were taken at 18°C. The percentages of resistance were calculated on the basis of the net resistance in sea water at the beginning of the experiment.

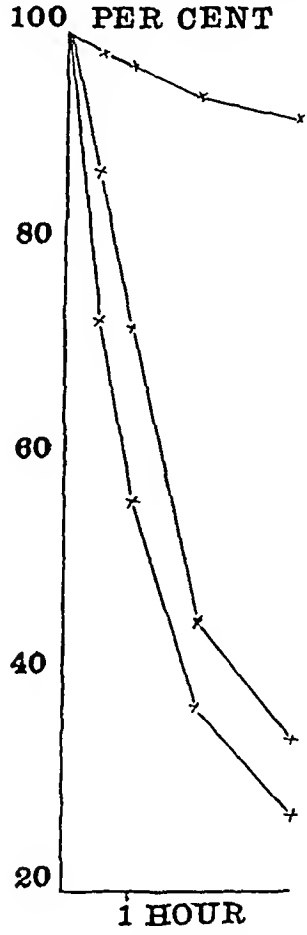


CHART 4. Curves of electrical resistance of *Laminaria saccharina* in NaCl 97.2 per cent + $CaCl_2$ 2.8 per cent (upper curve), in NaCl 97.2 per cent + $Ca(OH)_2$ 2.8 per cent (middle curve) and in NaCl (lowest curve).

In spite of the fact that the concentration of calcium ions was practically the same in the two solutions the behavior of the tissue was markedly different. In pure $CaCl_2$ the net resistance rose to 171 per cent of the original net resistance, while in $CaCl_2 + Ca(OH)_2$ it rose to only 113 per cent. At the end of forty-five minutes the resistance in $CaCl_2$ was 146 per cent, while in $CaCl_2 + Ca(OH)_2$ it was only 23 per cent.

These experiments make it evident that small amounts of NaOH are able to produce a considerable increase of permeability.

Loeb and Wasteneys,[6] who repeated Warburg's experiments, came to the conclusion that the very low concentrations of NaOH (0.001M and weaker) employed by him have practically no effect on oxidation and that the higher concentrations which are necessary to promote oxidation do so by injuring the egg. It will be seen that the results of the experiments described above are in harmony with this conclusion; since 0.001 M NaOH has practically no effect on permeability, while the stronger concentrations employed injure the cells if the exposure be sufficiently prolonged (but not when the exposure is short). It is, of course, possible that there are cells which are more sensitive to the action of alkali.

[6] This *Journal*, xiv, p. 459, 1913.

GASTRO-INTESTINAL STUDIES III (STUDIES ON WATER DRINKING XXI).

DIRECT DEMONSTRATION OF THE STIMULATORY POWER OF WATER IN THE HUMAN STOMACH.

By OLAF BERGEIM, MARTIN E. REHFUSS and PHILIP B. HAWK.

(From the Laboratory of Physiological Chemistry of Jefferson Medical College.)

(Received for publication, September 17, 1914.)

INTRODUCTION.

Heidenhain[1] was perhaps the first investigator to demonstrate that the flow of gastric juice was increased when water was introduced into the stomach, a view which was later confirmed by Sanotskii.[2] A much more firm experimental foundation for this important scientific and practical fact was furnished somewhat later by Pawlow[3] and collaborators. The great majority of these experiments were made on dogs equipped with the Pawlow pouch. It was demonstrated in these tests that the introduction of 400-500 cc. of water into the main stomach of the dog caused a secretion of gastric juice in the pouch, although the flow of juice was not abundant. In about 50 per cent of their tests no trace of gastric juice was secreted when 100-150 cc. of water were introduced. On the basis of such data they concluded: "It is only a prolonged and widely spread contact of the water with the gastric mucous membrane, which gives a constant and positive result." In these experiments all influences of a psychic nature were eliminated through the severing of the vagus nerves. Krshyschkowsky[4] demonstrated that water was ineffective as a gastric stimulant when introduced into the fundus of a dog's stomach through a

[1] *Arch. f. d. ges. Physiol.*, xix, p. 148, 1879.
[2] *Arch. Sci. biol.*, i, p. 588, 1892.
[3] *The Work of the Digestive Glands*, trans. by Thompson, London, 1910.
[4] Dissertation, St. Petersburg, 1906.

gastric fistula, without the knowledge of the animal. Lönnquist,[5] also working with dogs equipped with the stomach pouch according to Pawlow, demonstrated that the introduction of 200 cc. of distilled water into the main stomach brought about the secretion of gastric juice in the pouch. The total secretion for a period of two hours amounted to 5.43 cc. and possessed an acidity of 0.46 per cent HCl. The volume of fluid obtained from the large stomach aggregated 296.7 cc. and possessed an acidity of 0.22 HCl. After making proper correction for mucus, etc., it is evident that the 200 cc. of distilled water were instrumental in stimulating gastric secretion to an appreciable degree.

Foster and Lambert[6] confirmed the findings of Pawlow and collaborators previously mentioned when they observed that volumes of water below 200 cc. exerted no appreciable or uniform stimulation upon gastric secretion. They also observed, as did Pawlow, that larger volumes of water possessed a stronger stimulatory power. Taken as a whole, their experiments emphasize water as a gastric stimulant more than do those of Pawlow. According to Foster and Lambert, the increase in the flow of gastric juice which follows the introduction of water is directly proportional to the volume of water employed. This point is shown in the following data taken from one of their tests.

300 cc. water = 7.2 cc. gastric juice.
500 cc. water = 17.7 cc. gastric juice.
750 cc. water = 25.7 cc. gastric juice.

A similar proportionality between the volume of water employed and that of the gastric juice secreted had previously been shown by Chighin.[7] This investigator showed that a volume of 2.1 cc. of gastric juice was secreted by the isolated pouch upon introducing 150 cc. of water into the main stomach, whereas the introduction of 500 cc. yielded 7.2 cc. of juice. The acidity and digestive power of the juice were the same in each instance.

Sawitsch and Zeliony[8] have recently demonstrated a flow of

[5] *Skand. Arch. f. Physiol.*, xix, p. 220, 1906.
[6] *Journ. of Exp. Med.*, x, p. 820, 1908.
[7] Dissertation, St. Petersburg, 1894.
[8] *Arch. f. d. ges. Physiol.*, cl, p. 123, 1913.

gastric juice in the fundus following the introduction of water into the pyloric portion of the stomach. Carlson, Orr and Brinkman[9] in their experiments on cats also show water to be a gastric stimulant. They say: "These experiments certainly demonstrate the favorable action on gastric secretion of water taken with or after meals." Such favorable action has been repeatedly demonstrated.[10]

The experiments by the various investigators thus far mentioned were without exception performed upon lower animals. That water acts as a gastric stimulant in the *human* organism has been demonstrated by Wills and Hawk.[11] In these tests the ingestion of water *at meal time* by two men was accompanied by an *increase in the excretion of ammonia.* Inasmuch as certain experiments on lower animals had demonstrated that water causes an increased flow of gastric juice and since certain other experiments had demonstrated that the formation of acid in the body or the introduction of acid from without produced an increase in urinary ammonia, the authors felt justified in assuming that the increase in the ammonia excretion observed in their experiments was due to the stimulation of gastric secretion by the ingested water. They confirmed the observations of Chighin and of Foster and Lambert as to direct proportionality between the volume of the ingested water and that of the secreted gastric juice. This relationship of exact proportionality was not obtained in later experiments made by Wilson and Hawk[12] under similar conditions. Evidence to be submitted by us in the present paper makes it appear that the obtaining of such an exact relationship, as far as the human organism is concerned, is very problematical.

Edkins[13] claims to have shown that "Normal saline solution introduced into the stomach would remain for a prolonged period

[9] *Amer. Journ. of Physiol.*, xxxiii, p. 86, 1914.

[10] Fowler and Hawk: *Journ. of Exp. Med.*, xii, p. 388, 1910; Mattill and Hawk: *Journ. Amer. Chem. Soc.*, xxxiii, p. 1929, 1911; Hattrem and Hawk: *Arch. of Int. Med.*, vii, p. 610, 1911; Blatherwick and Hawk: *Biochem. Bull.*, iii, p. 28, 1913; Sherwin and Hawk: *Journ. Amer. Chem. Soc.*, xxxvi, p. 1779, 1914.

[11] *Proc. Amer. Soc. Biol. Chem.*, ii, p. 23, 1910; also *Journ. Amer. Chem. Soc.*, xxxvi, p. 158, 1914.

[12] *Journ. Amer. Chem. Soc.*, xxxvi, p. 1774, 1914.

[13] *Journ. of Physiol.*, xxxiv, p. 133, 1906.

(1–2 hours) unabsorbed and without change in reaction." Edkins worked on anesthetized cats.

METHODS.

The purpose of the present investigation was to study the stimulatory power of water in the human stomach, by a *direct* method. The subjects of the tests were normal men. In some experiments distilled water was used whereas in others the regular city water supply was utilized. In all our tests the Rehfuss tube was employed. This has been described elsewhere.[14] It is a stomach tube somewhat similar in character to the Einhorn duodenal tube. It differs materially from the latter in possessing a heavier tip which bears large perforations thus permitting the withdrawal of samples of gastric juice with great facility. These samples are removed by aspiration. The tip may be left in the stomach for hours without causing the slightest inconvenience.

Some of our tests were made early in the morning (empty stomach) whereas others were begun after an interval of two hours or more following an Ewald meal. After causing the subject of the test to swallow the tip (which is very easily accomplished because of its weight) the desired volume of water (50–500 cc.) was introduced into the stomach by drinking or through the tube by means of a syringe. Specimens of the stomach contents were then removed at intervals. The samples as removed were analyzed for total acidity (phenolphthalein), free acidity (Sahli)[15] and peptic activity (Mett).

Ordinarily about 10 cc. of gastric juice were withdrawn for analysis. If necessary the specimens were filtered before analysis. The filtration was not necessary in the water tests. Care was always taken to collect the saliva of the subject in order to guard against the contamination of the gastric juice by swallowed saliva. In determining total acidity 1 cc. of juice was titrated with $\frac{N}{100}$ NaOH, with phenolphthalein as an indicator, and the acidity values were expressed as cc. of $\frac{N}{10}$ NaOH necessary to neutralize 100 cc. of juice. In Sahli's method for free acidity 1 cc. of the juice was treated with 1 cc. each of potassium iodide (48 per cent) and

[14] Rehfuss: *Amer. Journ. Med. Sci.*, cxlvii, p. 848, 1914.
[15] Sahli: *Diagnostic Methods*, 1913.

potassium iodate (8 per cent) and titrated with $\frac{N}{10}$ sodium thiosulphate, with starch as an indicator. The free acidity values were also expressed in terms of $\frac{N}{10}$ KOH.

The Mett tubes used in determining peptic activity were made as suggested by Christiansen.[16] The tubes were placed in juice diluted with ten volumes of 0.2 per cent HCl and left at 37°C. for twelve hours. Peptic activities are expressed in millimeters squared.

EXPERIMENTS.

Our investigation into the rôle of water as a gastric stimulant has been conducted along several different lines. The number of individual experiments has therefore been rather large. For the most part our discussion will be limited to single experiments which are illustrative of the different phases of our data.

Experiment 14. This experiment was conducted upon Subject H, a man who had a previous history of hyperacidity, but who had experienced no inconvenience from this source for a period of at least two years. He was given a regulation Ewald meal and after an interval of two and one-half hours when the stomach was approximately empty, he drank 200 cc. of ordinary city water. Samples were withdrawn from the stomach at intervals of ten minutes for a period of two hours. The data from the analyses of these samples are shown graphically in Figure I.

The data from this test indicate that the acidity rose progressively for seventy minutes after the water entered the stomach. The total acidity at this point was 70 and the free acidity 60. From this point the acidity decreased and underwent a secondary rise in the latter part of the period. These data furnish a clear demonstration of the stimulatory power which water exerts over the activity of the gastric glands of the human stomach.

The peptic activity increased progressively from the beginning to the end of the test (see Experiments 21 and 60). Boldyreff[17] would probably explain this observation of increasing peptic activity with decreasing acidity as due in part to the neutralization brought about by regurgitation. The regurgitated pan-

[16] *Biochem. Zeitschr.*, xlvi, p. 257, 1912.

[17] *Quart. Journ. Exp. Physiol.*, viii, p. 1, 1914.

creatic juice would undoubtedly influence the acidity to a greater degree than it would influence the peptic power.

Experiment 16. Subject of the experiment, O.B., a normal man with no gastric history. Ewald meal administered and three and one-half hours later the subject drank 400 cc. of city water. Data are represented graphically in Figure II.

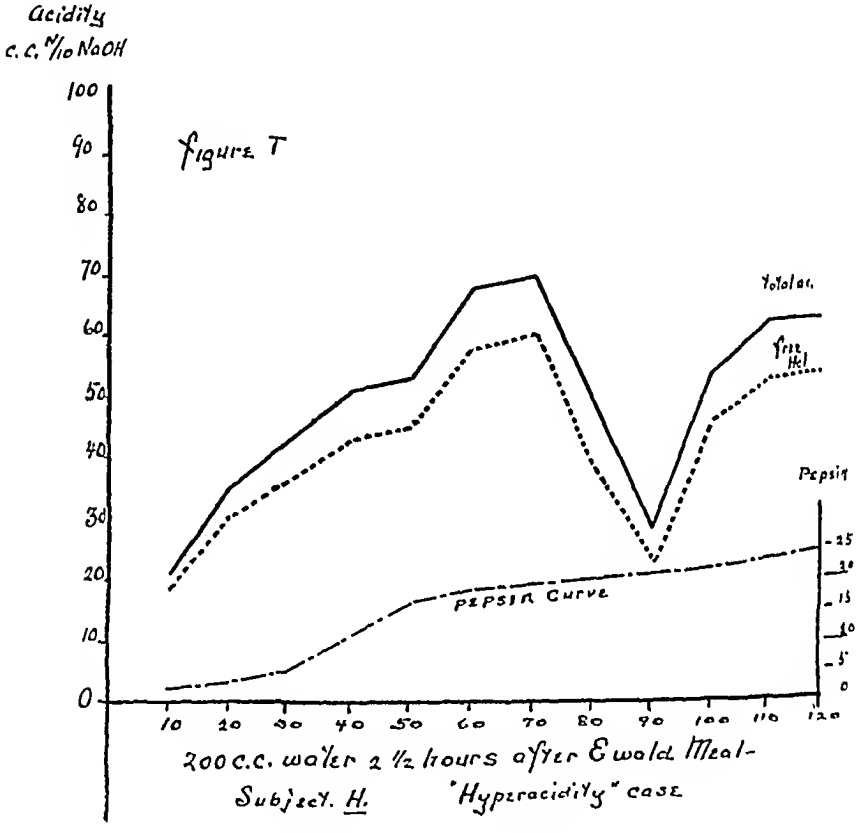


FIGURE I. *Experiment 14.*

The data in Figure II indicate that 400 cc. of water stimulated the activity of the gastric glands of this normal man sufficiently to produce an acidity of 66.0 in an interval of only one-half hour. From this point the acidity decreased and was represented by a value of 24.5 at the end of one hour from the time that the water was introduced. It is worthy of note that the water possessed approximately the same stimulatory power in this case as in the case of the subject with the hyperacidity history (Subject H in Exp.

14). In the case of the hyperacidity subject the maximum acidity was 70, a value obtained after an interval of one hour, whereas in the case of the normal subject in Experiment 16, the maximum acidity was 66 and was obtained in one-half hour. The data from the Ewald meals of these two subjects showed an acidity of 83 for the hyperacidity case and 63 for the normal subject. Atten-

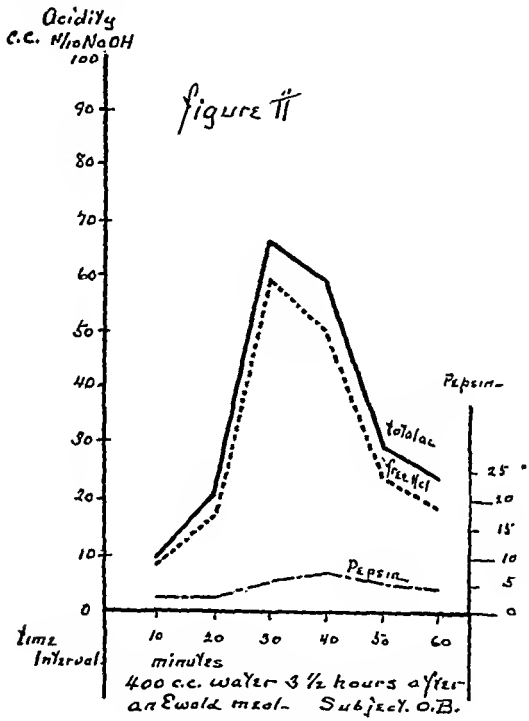


FIGURE II. *Experiment 16.*

tion should be called to the fact that the acidity value in the latter instance (normal subject) was lower after the Ewald meal (63) than after water alone (66), whereas in the case of the hyperacidity subject the reverse was true. That the maximum acidity was reached sooner in Experiment 16 than in Experiment 14 may be due to the fact that the stomach of the hyperacidity subject may evacuate more slowly than the normal stomach, thus per-

mitting the factor of *dilution* to postpone the appearance of the maximum acidity. This may be true notwithstanding the fact that the volume of water used in Experiment 14 was only one-half (200 cc.) that used in Experiment 16 (400 cc.). Experiments to be described in this series indicate that the acidity of the juice which is secreted in response to the water stimulus is in no way regulated by the volume of the water employed.

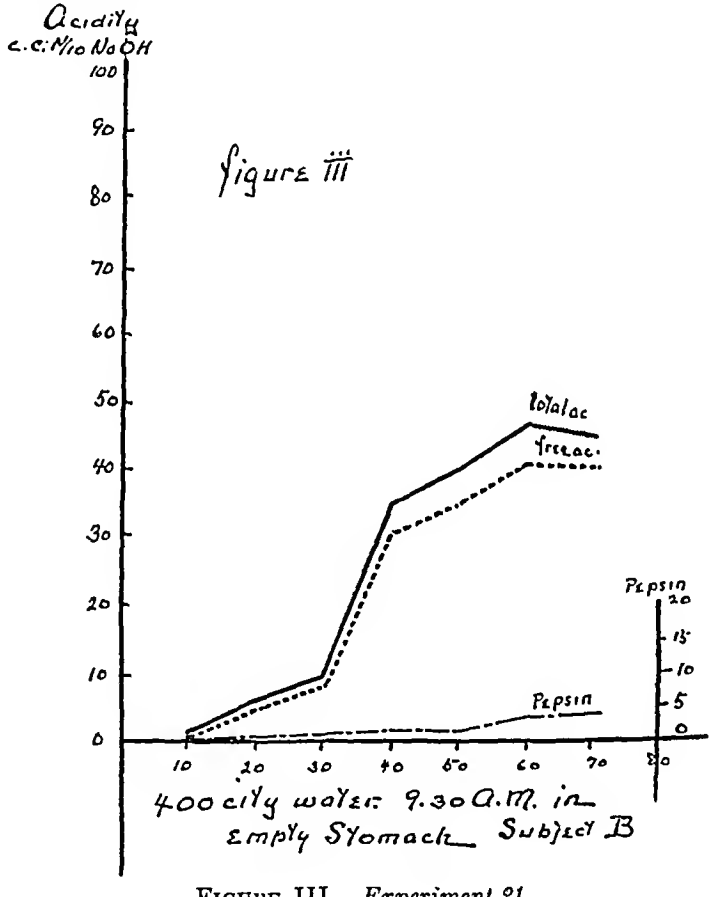


FIGURE III. *Experiment 21.*

The peptic activity increased to a maximum 40 minutes after the water was taken, then decreased in the remaining periods.

Experiment 21. Subject of the experiment, B. Normal and without gastric history. Given 400 cc. city water at 9.30 a.m. (see Fig. III).

The data in Figure III show that 400 cc. of city water taken in the morning before breakfast caused a gradual increase in the acidity of the gastric juice for a period of one hour, at which point a maximum acidity of 46.5 was registered. This experiment is one of the few cases in which the acidity of the first sample withdrawn from the stomach was entirely due to *free* acid.

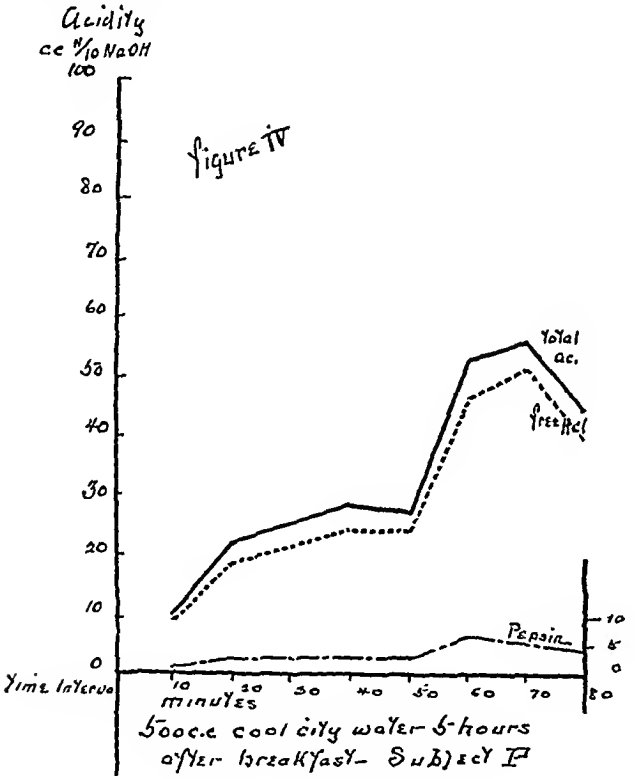


FIGURE IV. *Experiment 23.*

As a rule, despite the fact that the stimulus used was water, it was remarked that the total acidity and free acidity were not identical. This may be due in part to a partial neutralization or conversion of the free acid into combined acid by its passage over the mucosa.

The peptic activity values were rather lower in this experiment than in those which preceded it. The values increased progressively from the beginning to the end of the test (see Expts. 14 and 60).

Experiment 23. Subject P. Normal adult. Negative gastric history. 500 cc. cool (10°–12°C.) city water were taken at noon, five hours after the last meal. For data see Figure IV.

The above data indicate that 500 cc. of cool city water taken by a normal man five hours after breakfast caused sufficient stimulus to produce an acidity of 56.5 at the end of seventy minutes. This

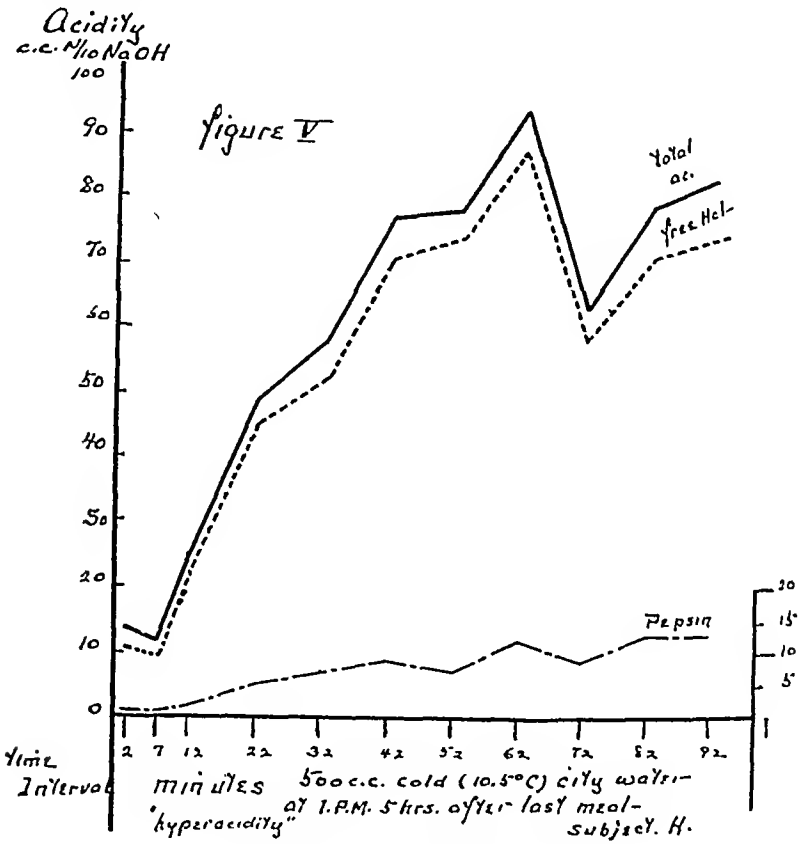


FIGURE V. *Experiment 27.*

stimulation is entirely comparable to that indicated in Experiment 21.

The peptic values increased to a maximum at one hour, then decreased.

Experiment 27. This experiment was conducted on the hyperacidity subject previously used in Experiment 14. In this instance the subject was given 500 cc. of cold city water (10.5°C.) at 1 p.m., five hours after the last meal (see Fig. V).

The data indicate that a total acidity of 94.5 and a correspondingly high free acidity value (88) were obtained one hour after the drinking of the water. These acidities are higher than those obtained after the Ewald meal given in Experiment 14 or after the ingestion of water which followed the Ewald meal in that experiment. The total aciditics in the three cases were as follows: Ewald meal = 83; water two and one-half hours after Ewald meal = 70; water five hours after an ordinary mixed breakfast = 94.5.

This experiment marked the most pronounced water stimulation obtained thus far in the series of tests, as far as acidity values are concerned. The peptic values were not so high as those in the first test with this subject (see Experiment 14).

Experiment 29. This experiment was conducted with a desire to secure data as to "gland fatigue," to which reference has been made by Foster and Lambert[18] and others. A normal man, S. McD., was given 500 cc. city water (10°–12°C.) at 1 p.m., five hours after breakfast, and samples of juice were collected at ten-minute intervals until the stomach was approximately empty. After an intermission of ten minutes the experiment was repeated. The data are charted in curve form in Figure VI.

The total acidity ten minutes after the first 500 cc. of water were taken was 4.5. This value increased progressively and reached 49.5 at the end of forty minutes. The second volume of 500 cc. of water produced a total acidity of only 1.0 at the end of ten minutes and one of 25 at the end of forty minutes. In the next ten minutes, however, the acidity reached 45.0, a value very closely comparable with the acidity (49.5) obtained forty minutes after the first 500 cc. of water were ingested. These data may be interpreted as indicating a slight glandular fatigue. However, this fatigue is apparently not so pronounced as has been claimed. The curves (Fig. VI) represent the similarity of the response obtained with the same stimulus in the same individual and tend to confirm other studies which we have made demonstrating that under identical conditions an individual always reacts in the same way.

[18] *Journ. of Exp. Med.*, x, p. 820, 1908.

Experiment 30. This experiment was made on J. T. L., a normal man with no gastric symptoms. He was a pronounced smoker. The subject received 500 cc. of cool (10°–12°C.) city water at 1 p.m., six hours after the last meal. In other words, the experimental conditions were the same as those in Experiment 27 and in the first half of Experiment 29 (see Fig. VII).

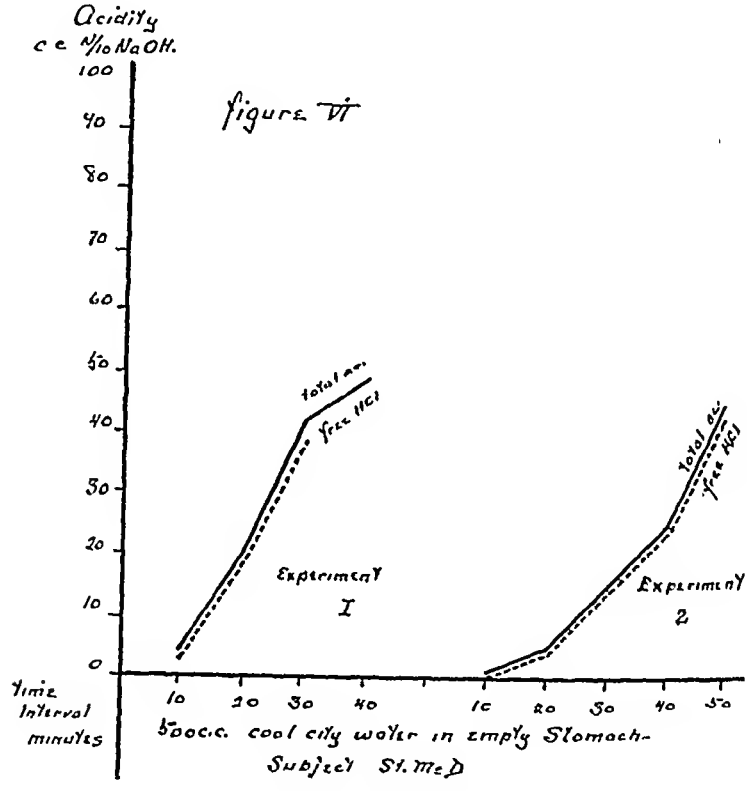


FIGURE VI. *Experiment 29.*

The data from this experiment furnish the most striking illustration of the stimulatory power of water which our investigation affords. Ten minutes after the water had passed into the stomach the total acidity was 19.0, and ten minutes later this acidity value had jumped to the surprisingly high value of 111.5. The succeeding values showed but slight variation from this value. In other words, the water possessed such a remarkable stimulatory power as to cause an acidity of over 100 in an interval of less than twenty minutes.

We believe that the data from this test also furnish evidence of the rapidity with which the water left the stomach. We may believe that the 500 cc. water upon reaching the stomach at once stimulated the gastric glands to greater activity, and caused the contents of the stomach to assume an acidity of 19.0. Some time

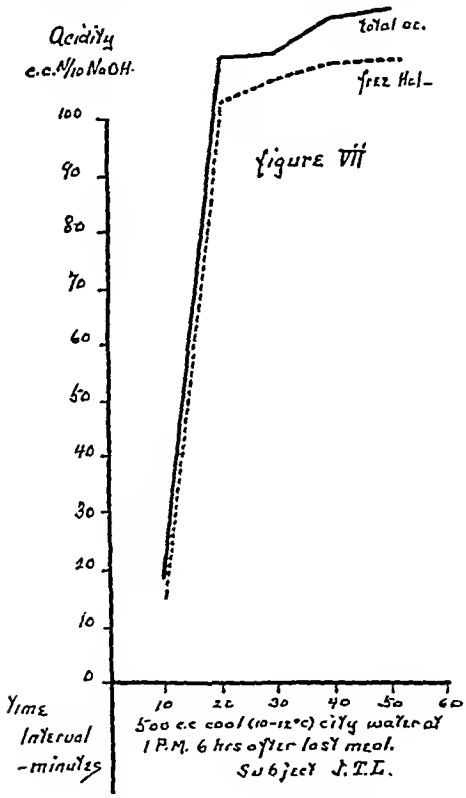


FIGURE VII. *Experiment 30.*

during the next ten minutes, *i.e.*, ten to twenty minutes after the water first reached the stomach, practically the entire 500 cc. had passed into the intestine and left behind a gastric juice of very high acid concentration (111.5). That the stomach was practically empty in from 10–20 minutes, as far as the original water was concerned, is indicated by the uniform values obtained for acidity

in the samples withdrawn from the stomach during the next half hour. In other words, we believe that the only acidity value which was influenced by the factor of dilution was the acidity value of the ten minute sample. Some time before the next specimen was taken the large volume of water had passed into the intestine and our acidity value (111.5) represents the true stimulatory power of the water unmasked by the factor of dilution. This is an example of the hypersecretory type which we have discussed in a former paper[19] and represents the tendency to an exaggerated response to a constant stimulus both as to the height of acidity and the quantity of the secretion.

X-ray studies have thrown interesting light on the evacuation of known quantities of water. 200 cc. according to Holzknecht[20] are evacuated normally in sixty minutes. A somewhat similar statement is made by Kaestle,[21] who claims that 200 cc. of water leave the stomach normally in seventy minutes (extremes; fifty-five to eighty minutes), whereas in pyloric stenosis and atony this limit is exceeded. But this does not take into account the question as to whether the water or the secretion induced by it leave the stomach in that time. The general concensus of opinion is that water leaves the stomach rapidly, the bulk of it in the first few minutes along the so called "Rinne," or trough, in the lesser curvature, this being particularly true of the empty stomach. Waldeyer[22] and Kauffmann[23] established the presence of this trough on anatomical grounds, Ernst[24] contributed evidence from a pathological standpoint, and Cohnheim[25] apparently succeeded in directly observing this phenomenon in his experiments on dogs. Scheunert,[26] on the other hand, takes the opposite view and claims from his experiments on the horse's stomach, that liquid in the distended stomach has a tendency to permeate along the gastric walls.

[19] Rehfuss, Bergeim and Hawk: *Journ. Amer. Med. Assoc.*, lxiii, p. 909, 1914.

[20] *Wiener klin. Wochenschr.*, Nos. 32 and 33, 1913.

[21] *Rieder's Röntgenkunde*, Leipsig, 1913, p. 532.

[22] *Berliner Akademie*, 1908, cited by Gröbbels: *Zeitschr. f. physiol Chem.*, lxxxix, p. 1, 1914.

[23] *Zeitschr. f. Heilk.*, xxviii, p. 203, 1907.

[24] Cited by Cohnheim: *Münch. med. Wochenschr.*, liv, p. 2581, 1907.

[25] *Münch. med. Wochenschr.*, liv, p. 2581, 1907.

[26] *Arch. f. d. ges. Physiol.*, cxliv, p. 411, 1912.

The effect of water combined with foodstuffs has also been the subject of interesting experiments. Gröbbels[27] is authority for the statement that in dogs the digestion of bread followed by water is shorter than that of bread alone. Gabrilowitch[28] demonstrated that in the administration of a mixture of meat and water the water passes out of the stomach allowing the meat to follow its customary digestion. Somewhat larger quantities of water require a longer time and slow the digestion proportionately.

Experiment 34. Our experiments thus far had entailed the use of water from the city supply. We were, therefore, unable to determine whether the stimulatory power of the water was due to contained electrolytes or to the water *per se.* For this reason our experiments from this time forward were made with *distilled water.*

In Experiment 34 S. McD. served as subject. He was given 500 cc. cool distilled water at 1 p.m., about six hours after breakfast. Data are given in Figure VIII.

The subject of this experiment was also used in Experiment 29, when city water was employed. A comparison of the data is therefore of interest. In the first part of Experiment 29 the ingestion of 500 cc. of city water produced a total acidity of 49.5 in forty minutes. In Experiment 34 the ingestion of 500 cc. of distilled water produced a total acidity of 65.5 in forty minutes and one of 86.0 in fifty minutes. In other words the gastric glands of this subject were apparently stimulated in a more pronounced manner by pure distilled water than they were by the city water with its mixed electrolyte content. We have not made a sufficient number of comparative tests, however, to claim that this is uniformly true.

An earlier experiment on this same subject in which an Ewald meal was given indicated a total acidity of 53.5 one hour after the meal. In the present experiment 500 cc. of distilled water yielded a total acidity of 86.0 in fifty minutes. The greater stimulation of the water over that of the Ewald meal was demonstrated with certain of our other subjects. In one other case (Exp. 11) with subject S. the Ewald total acidity was 54, whereas a later test (Exp. 35) with 500 cc. of distilled water showed a total acidity of 81.

[27] *Zeitschr. f. physiol. Chem.*, lxxxix, p. 1, 1914.
[28] *Ibid.*, lxxxi, p. 398, 1912.

These experiments demonstrate and constitute a potent argument for a water test meal. The great advantage of such a procedure would be the demonstration of food rests, the finer particles of which are often lost in the body of an Ewald meal.

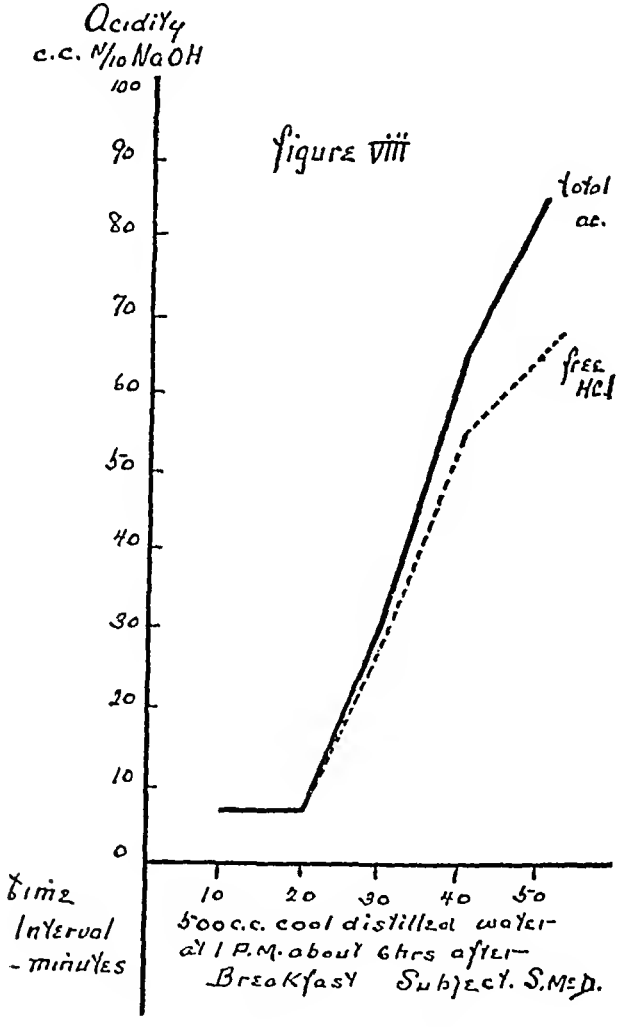


FIGURE VIII. *Experiment 84.*

Experiment 87. This experiment was made using Lh., a normal man, as subject. He was given 100 cc. distilled water at 9.30 a.m., having had no food since dinner the day before. The tests thus far having been made with *cold* water, we planned to make some

tests with warmer water. The temperature of the water in the present instance was 50°C. (see data in Fig. IX).

The maximum acidities were 56 (total) and 49.5 (free), which appeared forty minutes after the water was introduced.

Experiment 38. This experiment was the same as Experiment 37 except that Ls. was used as a subject. This man was normal

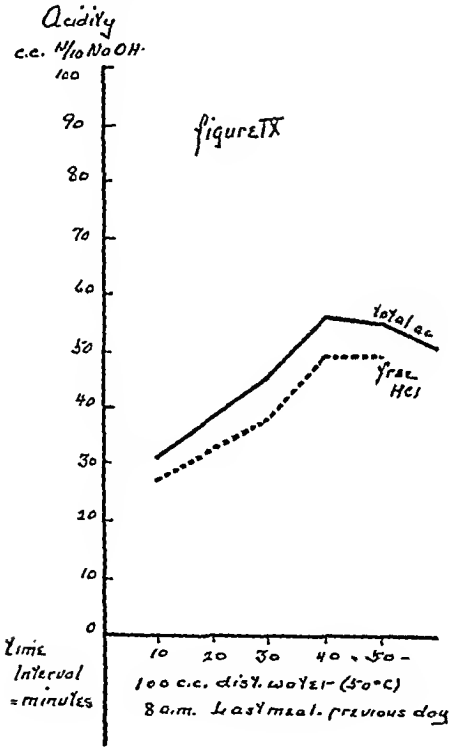


FIGURE IX. *Experiment 37.*

and had no gastric symptoms. However, three years previously he was troubled with hyperacidity. Data are charted in Figure X.

In this instance the water acted as a very strong stimulus and caused acidities of 107.5 (total) and 94.5 (free) in fifty minutes. These very high values when compared with the much lower values obtained in Experiment 37 under identical conditions,

serve to emphasize the factor of *individuality*. The 100 cc. of warm water had 100 per cent greater stimulatory power in the stomach of Ls. than in the stomach of Lh. The hyperacid tendency of the former, whether due to vagotomy or an irritable mucosa, may have been a contributing factor.

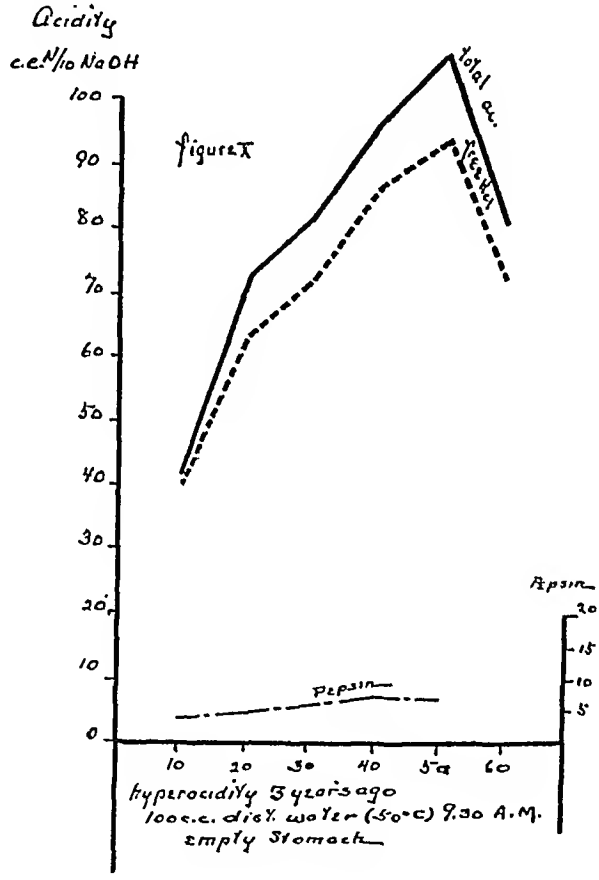


FIGURE X. *Experiment 38.*

The peptic activity reached the maximum in forty minutes, or ten minutes before the acidity reached its highest point.

Experiment 48. In this test subject J. was first given 50 cc. of bouillon (40°C.), and after the secretion of juice had apparently ceased following the extractive stimulation, 50 cc. of distilled water (40°C.) were passed into the stomach *by the tube*. In previous experiments the subjects *drank* the water (see data in Fig. XI).

The data indicate that the previous stimulation by meat extract had apparently not fatigued the gland to any degree. This would seem to follow from the fact that a total acidity of 100.5 was obtained twenty minutes after the 50 cc. of water were passed into the stomach. This was a somewhat higher acidity than that obtained by the bouillon stimulation (94.9).

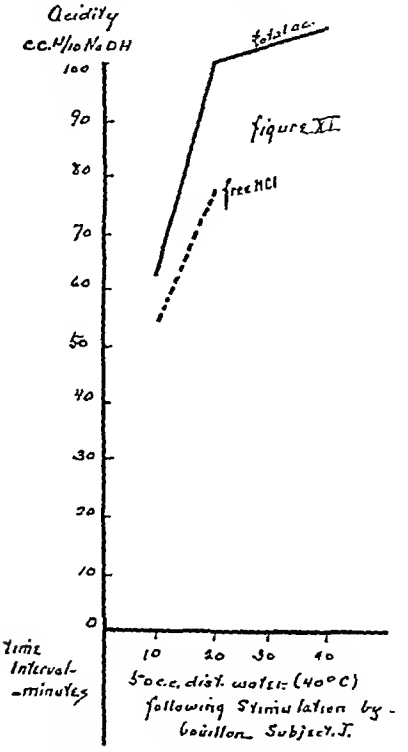


FIGURE XI. *Experiment 48.*

Experiment 40. In this experiment subject Hn. received 50 cc. of distilled water (50°C.) at 1 p.m., about six hours after the last meal (see Fig. XII).

The above data constitute the most pronounced instance of "continued digestive secretion"[29] which our experiments afford in

[29] Cf. Rehfuss, Bergeim and Hawk: *Journ. Amer. Med. Assoc.*, lxiii, p. 11, 1914.

normal individuals. It is certainly surprising that 50 cc. of distilled water should cause sufficient stimulus to insure the outpouring of such volumes of juice of high acidity. The maximum acidity was 62.5 obtained at the end of three hours, whereas the most copious flow of secretion was one of 20 cc. obtained during

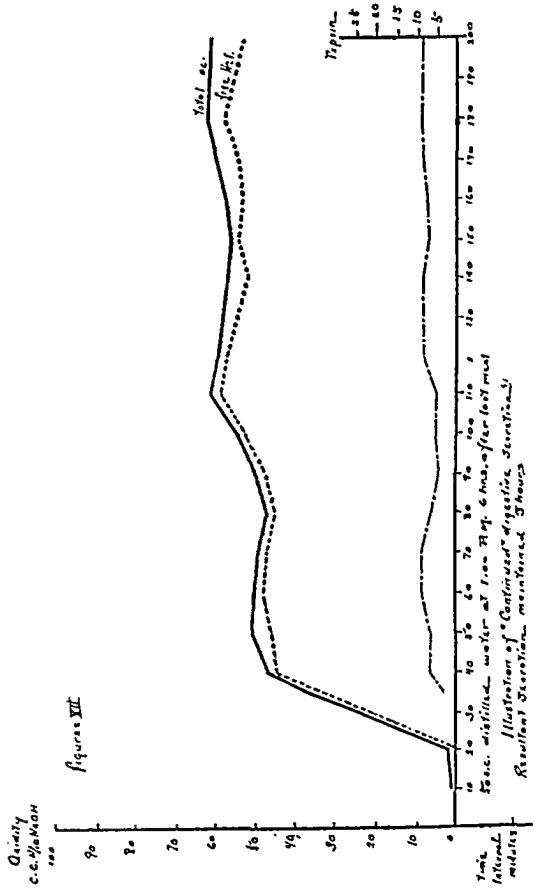


FIGURE XII. *Experiment 40.*

the last ten-minute period. The flow of the secretion was very uniform (11–14 cc. per period) from fifty minutes to one hundred and ninety minutes after the water was introduced into the stomach. The data from this test afford a marked contrast to those from Experiment 48 in which the same volume (50 cc.) of water was employed. In Experiment 48 the rise in acidity was very rapid, reaching the very high value of 106.5 in forty minutes,

at which time the stomach was approximately empty and with no apparent sign of "continued gastric secretion." In Experiment 40, on the other hand, the acidity rose slowly and did not reach its maximum for three hours, during which interval a total volume of at least 225 cc. of gastric juice was secreted as against 17 cc. in Experiment 48. In these experiments, it was amply demonstrated that even 50 cc. of water were capable of producing distinct stimulation. From the X-ray studies of Leven and Barret[30] we know that in a normal subject the stomach in the empty condition is in the state of a more or less contracted tube. The introduction of but 30 cc. of liquid is sufficient to fill the organ to the level of the air chamber. It must therefore be evident that even this small amount is capable of coming in contact with practically every part of the mucosa.

The peptic activity increased irregularly to a maximum at the end of the experiment.

Experiment 52. At this point in our experiments the possible influence upon our findings of the morning gastric residuum[31] was considered. Experiments from this point were made after the removal of the residuum.

In this particular experiment S. served as a subject. The residuum was removed by aspiration and the contents of the stomach removed at intervals of a few minutes until the organ was empty. At this point 50 cc. distilled water (40°C.) were introduced by the tube. The data are shown in Figure XIII.

The data show a maximum acidity of 53.5 which was obtained at the end of fifty minutes. The residuum removed had a volume of 97 cc. and a total acidity value of 35.5. Had it been present in the stomach when the water was introduced it is possible that the acidity values as determined would have been considerably altered from those actually obtained. The presence of the residuum would prevent the water from coming into such wide-spread contact with the stomach mucosa as would be possible in the empty stomach, a fact which would tend to lessen the stimulatory power of the water.

[30] Leven and Barret: *Radioscopie gastrique*, Paris, 1909, p. 65.

[31] Rehfuss, Bergeim and Hawk: *Journ. Amer. Med. Assoc.*, lxiii, p. 11, 1914.

In this case the peptic activity reached its maximum in forty minutes, or ten minutes before the maximum acidity was reached. The same order was observed in Experiment 38.

Experiment 60. The subject of this test was Hs. The residuum (60 cc.) was removed from the stomach at 9 a.m., and the organ aspirated at intervals of ten minutes for one hour and forty min-

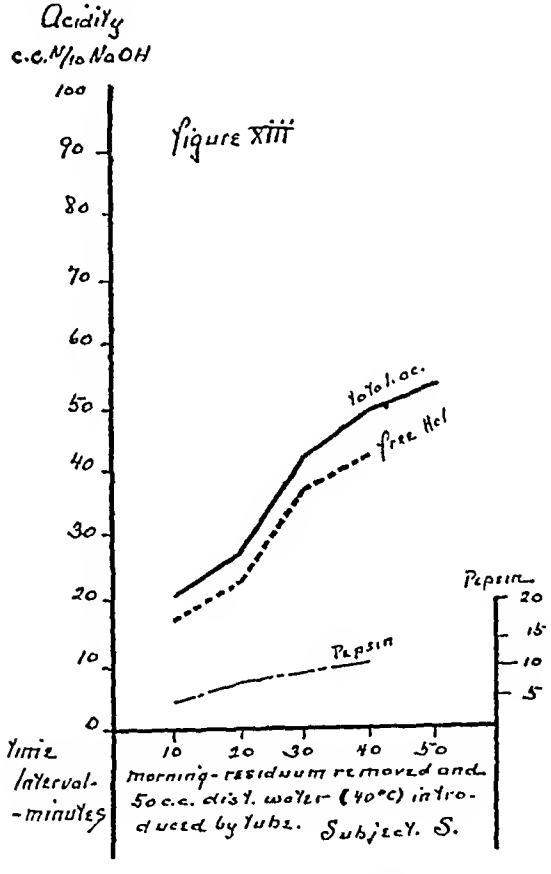


FIGURE XIII. *Experiment 52.*

utes. Lavage was then performed (acidity of wash water 5.0) and immediately afterward 200 cc. distilled water were introduced into the empty stomach by means of the tube. The data are given in Figure XIV.

The data indicate that the 200 cc. of distilled water introduced into the stomach after removal of residuum and subsequent lavage stimulated the gastric glands in a marked manner, yielding acidities

of 94.0 (total) and 73.0 (free) in forty minutes. It is probable that the acidity values were increased somewhat, due to the supplementary stimulation of the lavage water.

Here again we have the peptic activity increasing progressively from the beginning to the end of the test, as in Experiments 14 and 21.

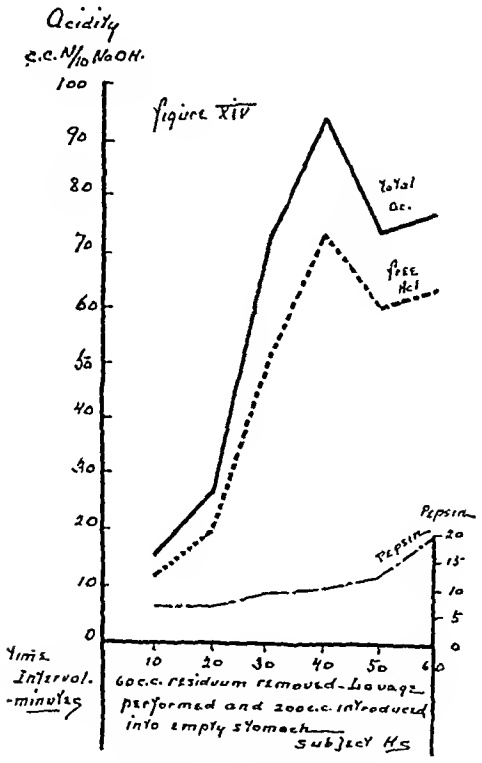


FIGURE XIV. *Experiment 60.*

Experiment 65. This experiment was conducted in order to obtain information as to the "latent period" of the human gastric glands. The subject was M., a normal man. Residuum removed at 8 a.m. The volume was 30 cc. and the total acidity 60. Lavage was then performed, the water removed having a total acidity of 7.0. At this point 100 cc. of distilled water were intro-

duced into the stomach through the tube. The data follow in Figure XV.

Pawlow[32] having shown that the latent period of the gastric glands of the dog is about five minutes it was considered of interest to determine the latent period of the human gastric glands. For this reason the samples were taken at *one-minute*

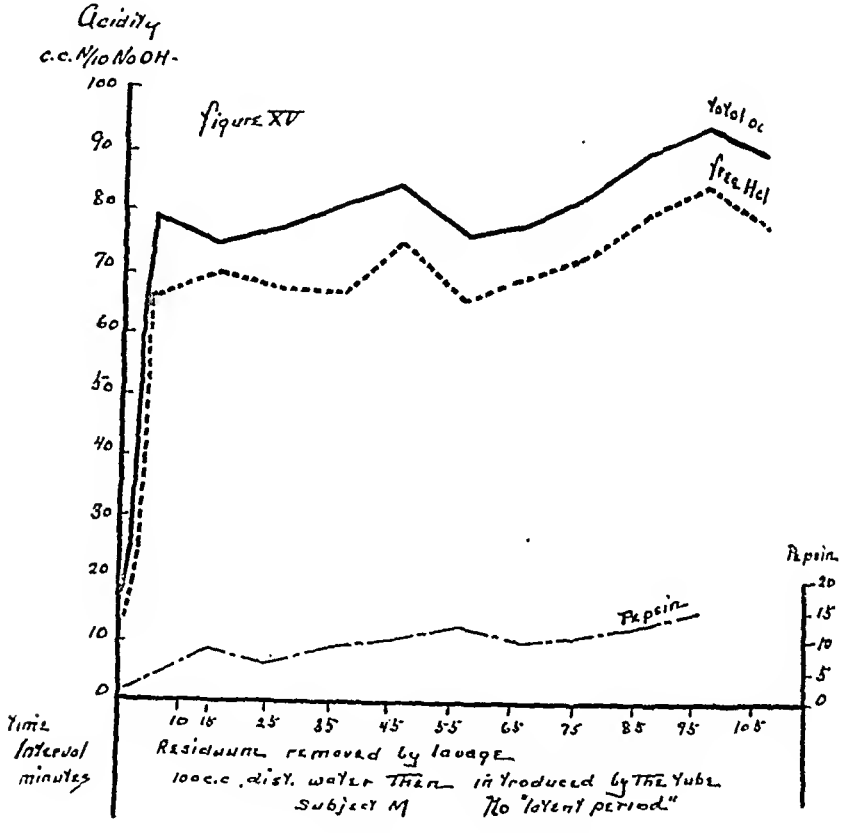


FIGURE XV. *Experiment 65.*

intervals for a five-minute period immediately after the introduction of the water. The data indicate very clearly that *there was no latent period.* The glands apparently became active as soon as the water reached them. This would seem to be demonstrated by the observation of total acidity values of 19.5, 26, 49.5, 63.5 and 79.5 during the first five minutes the water was in contact

[32] *The Work of the Digestive Glands,* trans. by Thompson, London, 1910.

with the gastric mucosa. We expect to do further work in the study of the latent period, but thus far our data seem to indicate that the five-minute latent period as determined by Pawlow for the dog does not hold for the human stomach.

In this experiment the Mett values increased for fifty-five minutes, at which point a value of 12.25 was recorded. Then came a slight falling off in the peptic activity followed by the maximum (15.21) at the end of the test.

It is very apparent that the data presented in this paper bearing upon the stimulation of the gastric glands of man do not harmonize with similar data obtained by Pawlow and others from animal experimentation. We believe that this fact indicates that some at least of the current ideas regarding gastric stimulation which are based upon data secured from experiments upon Pawlow animals must be revised as soon as sufficient evidence has accumulated from direct experiments upon the human subject, such as are outlined in the present paper. Take the question of gastric stimulation, for example. Pawlow cites very definite evidence that 150–200 cc. of water in 50 per cent of his cases caused no stimulation of the flow of gastric juice. In our experiments on man, as small a volume as 50 cc. (we did not experiment with smaller volumes) caused in every instance a very distinct stimulation, as evidenced by increases in both acidity and enzyme values. Compared from the standpoint of body weight or unit area of gastric mucosa the failure of the dog to respond to water stimulation is all the more marked. In other words, the gastric glands of a dog whose body weight is only one-third to one-eighth that of a man and whose gastric area is proportionately small are not stimulated by a volume of water four times as great as that sufficient to cause pronounced stimulation of the gastric glands of the man.

One point must be borne in mind in making the above comparison. If we were to make direct tests upon dogs such as those outlined in this paper as made upon man, would the gastric glands of the dog still fail to respond to the stimulatory power of 150–200 cc. of water? Or, were we to experiment upon a human being possessed of a Pawlow pouch, would we still be able to demonstrate an increased flow of gastric juice from the pouch when 50 cc. or even 100–150 cc. of water were introduced into the main stomach? To questions such as these we have no answer. However, we are of

the opinion that the response to stimulation will be more pronounced when the stimulating factor comes into actual physical contact with the gastric mucosa, as in our experiments. In the experiments with Pawlow animals, the factor whose stimulatory power it is desired to study is placed in contact with the mucosa of the large stomach, whereas the stimulatory power of that factor is gauged by the response of the mucosa of the pouch. We are inclined to believe that the actual physical contact of the stimulating substance with the gastric mucosa is a necessary factor to the securing of the maximum stimulation. We have planned a series of tests on dogs and men which, we trust, will give us some information on this point.

CONCLUSIONS.

1. Water (ordinary or distilled), either cold (10.5°C.) or warm (50°C.), is a *very strong gastric stimulant* and in certain instances yields an acidity of over 100 (cc. $\frac{N}{10}$ NaOH to neutralize 100 cc. of juice), in less than twenty minutes.

2. As small a volume of water as 50 cc. has been demonstrated to have a pronounced and immediate stimulatory power in the human stomach.

3. In the average normal individual water produces fully as great a stimulation (as measured by acidity and enzyme values) as does an Ewald test meal, and the acidity values follow a similar type of curve. A simple water meal might therefore be substituted in many instances for the Ewald meal, and has the additional advantage of demonstrating any food rests.

4. It was impossible to demonstrate any pronounced glandular fatigue in the human stomach. Identical volumes of water introduced into the stomach of the same man during the same afternoon yielded very similar acidity values. The samples removed during the second test were but slightly lower in acidity than were those removed during the first test. The type of the curve was the same in each instance.

5. Under all conditions the increased acidity following water stimulation is accompanied by increased peptic activity, although the two types of values do not necessarily run parallel.

6. It was impossible to demonstrate any "latent period" for the human gastric glands. Therefore a "prolonged and wide-

spread contact with the stomach mucosa" is not a necessary preliminary to stimulation, as Pawlow and others have claimed. The introduction of 100 cc. of distilled water into a stomach made empty by lavage was followed by the removal of samples at one-minute intervals which showed total acidity values of 19.5, 26.0, 49.5, 63.5 and 79.5. The latent period of five minutes, as claimed by Pawlow for the gastric glands of the dog, therefore, probably does not hold for the stomach of man.

7. In one experiment definite evidence was obtained that 500 cc. of water left the stomach in ten to twenty minutes after its introduction (see Fig. VII).

8. In a single instance a very pronounced "continued digestive secretion" was observed. At least 225 cc. of gastric juice of relatively high acidity were secreted after an initial stimulus afforded by 50 cc. of distilled water (50°C.) (see Fig. XII).

9. Since water stimulates the gastric glands to activity when no food is present in the stomach as well as when there is a digestive task to complete, it would seem a waste of "glandular energy" to drink water between meals. It would seem that water could best further the digestive plan when taken *with meals.*

10. The stimulatory power of the water was not influenced in any uniform way by the volume of fluid introduced into the stomach. In some instances a small volume of water gave a pronounced stimulation, whereas in other tests the response was relatively less pronounced when a larger volume of water was employed.

11. Our acidity values emphasize again that the values (40–60) commonly accepted by clinicians as normal values for gastric acidity are undoubtedly too low. The acidity of the gastric juice secreted after water stimulation ranged from 50 to 120 (0.18–0.44 per cent HCl) with an average value of 77 (0.28 per cent HCl).

12. Our experiments made upon the human stomach by the fractional method lead us to conclude that at least some of the findings of Pawlow and others which have been obtained by animal experimentation cannot safely be considered as covering conditions existing in the human stomach. This is particularly apparent concerning the question of *stimulation.*

INFLUENCE ON GROWTH OF RATIONS RESTRICTED TO THE CORN OR WHEAT GRAIN.[1]

By E. B. HART and E. V. McCOLLUM.

(*From the Laboratory of Agricultural Chemistry of the University of Wisconsin.*)

(Received for publication, September 18, 1914.)

In 1911[2] there were published from this laboratory the records of the influence of restricted rations on the growth and reproduction of herbivora (cattle). These records show the inability of a "balanced" ration from the wheat plant to allow a complete cycle of life, while a balanced ration from the corn plant served this purpose admirably. Further records of experiments with the oat plant and a mixture of the above three rations were also given.

Since that time the work with herbivora has been continued and extended and will be made the subject of a future report. We have in addition studied the influence of the corn grain and wheat grain on the growth of swine and rats, and this paper will be confined to that work.

In recent years interesting experiments with restricted rations, made from naturally occurring food stuffs, have been carried out by Holst[3] and his co-workers in their studies on experimental scurvy. With guinea pigs, on a diet limited to certain grains or grain products, they were able to produce the characteristic condition of scurvy. This condition, however, could be corrected by the use in the diet of raw vegetables, or by a citric acid extract of the dried fresh material. The nature of the antiscorbutic substance is still unknown.

The more familiar example of beri-beri, a disease common in the Orient, is the result of feeding a restricted ration; namely,

[1] Published with the permission of the Director of the Agricultural Experiment Station.

[2] *Research Bulletin 17, Wisconsin Agricultural Experiment Station.*

[3] *Zeitschr. f. Hyg. u. infekt. Krankh.*, lxxii, p. 1, 1912.

polished rice. In this case it is believed by a number of investigators—Funk,[4] Suzuki[5]—that there is something removed in the polishing process which is absolutely necessary for the organism and which it cannot synthesize. This absence of a substance—the vitamine of Funk or oryzanin of Suzuki—is responsible, according to these investigators, for the condition of polyneuritis. On the other hand, Caspari and Maszkowski[6] are of the opinion that in the case of beri-beri we are dealing with a toxic substance in the seed and that this toxicity is overcome by antibodies present in the bran layers. The investigation of Miss Wheeler and Miss Biester[7] on the nutritive value of some proprietary infant foods is of very great interest in this connection. Certain of these materials made from milk were able to serve as complete foods for mice, but those prepared largely from grains were not capable of serving as an exclusive diet.

In connection with this consideration of the restricted *natural* diet it should be stated that the work of McCollum and Davis,[8] and later confirmed by Osborne and Mendel,[9] has established the necessity in the artificial diet for growth of some substance or substances not contained in purified proteins or carbohydrates supplemented with a suitable ash mixture. Further, the later work of Osborne and Mendel[10] on the nutritive value of purified proteins is establishing the limit of synthetic power of the animal cell for amino-acids. These are extremely important factors in the artificial diet, but it remains to be established whether they are ever complete limiting factors in the restricted but natural diet. Their proportion in the natural diet may be a very important factor in the *rate* of growth but we are referring here to any growth curve, whatever the rate may be.

These references were made for the purpose of again emphasizing the trend of nutrition investigations toward unexplained but very important fields and to point out the danger of restricted natural

[4] *Journ. of Physiol.*, xliii, p. 395, 1912.
[5] *Biochem. Zeitschr.*, xliii, p. 89, 1912.
[6] *Berliner klin. Wochenschr.*, l, pp. 1515–1519.
[7] *Amer. Journ. of Diseases of Children*, vii, p. 169, 1914.
[8] This *Journal*, xv, p. 167, 1913.
[9] *Ibid.*, xv, p. 311, 1913.
[10] *Ibid.*, xvii, p. 325, 1914.

diets, although we may have satisfied the energy, protein and mineral requirements of the organism. It is apparent from the views expressed that in restricted but natural rations we may be dealing in some instances with the absence of groups which the organism cannot synthesize, while in other cases toxic materials may be present to exert a slow but accumulative effect. Variety of source of diet, then, would mean the ample supply of all necessary groups for construction purposes, or the furnishing of antibodies which would counteract those toxic substances which may accompany even what we call a "normal" foodstuff.

In the artificial diet the factors of adequate protein, salt mixtures and unknown ether-soluble substances are important considerations in addition to the energy requirements.

EXPERIMENTS. (*Swine, 1912–13.*)

In these experiments strong healthy pigs of 40 to 70 pounds' weight were chosen and confined on floors and indoors. In order to provide dry sleeping berths, but to keep the bedding away from the animals, wire screens on frames were placed in one corner of the pen. Under these screens shavings were placed. This provision worked admirably and served as a dry clean place for the animals. *Distilled water was used throughout the work.* The rations were fed *ad libitum*, but only what would be entirely consumed. The animals were grade Poland China and were weighed weekly during the experiment. The rations in all cases were made up of natural products, but in certain instances a salt mixture was superimposed upon the corn and wheat rations. In two cases (rations 2 and 4) the salt mixture used was so constructed as to make the total mineral content of the ration approximately like that of a successful salt mixture used in artificial rations with rats. One hundred pounds of this rat ration contained the following ash constituents:

	grams		grams
CaO	172.3	P_2O_5	517.5
MgO	209.3	SO_3	210.5
K_2O	368.0	Cl	3.7
Na_2O	14.6		

To make the mineral content of rations 2 and 4 approximately

like the above the additions mentioned below were made. In both cases, however, no attempt was made to change the magnesium relation as it was thought high enough. In ration 6 the mineral mixture added was designed to furnish just sufficient quantity of bases to maintain the ration at the neutral point, for the reason that all grain rations have a preponderance of acid over basic elements. Of late a considerable amount of emphasis by various investigators, notably Sherman[11] and Forbes,[12] has been placed upon the balance of acid and base radicals in normal rations, it being held that the basic radicals should be sufficient to maintain neutrality or alkalinity in the tissues. These investigators apparently forget the very important function ammonia can play in preventing acid conditions in the tissues and the further fact that ammonia is abundantly supplied on ordinary rations from various sources in the animal as conditions arise requiring it.

The protein content of the rations was always raised to 14 to 15 per cent of the ration by the addition to the grain of a concentrate from the same kernel. The rations used were as follows:

Ration 1. 70 pounds corn meal; 30 pounds gluten feed.

Ration 2. 70 pounds corn meal; 30 pounds gluten feed; (323 grams K_2HPO_4; 513 grams Ca lactate).

Ration 3. 97.5 pounds wheat meal; 2.5 pounds wheat gluten.

Ration 4. 97.5 pounds wheat meal; 2.5 pounds wheat gluten; (259 grams K_2HPO_4; 36 grams K citrate; 684 grams Ca lactate).

Ration 5. 88 pounds wheat meal; 10 pounds wheat bran; 2 pounds wheat gluten.

Ration 6. 88 pounds wheat meal; 10 pounds wheat bran; 2 pounds wheat gluten; (276 grams Ca lactate; 338 grams K citrate; 317 grams Mg citrate).

Ration 7. 27 pounds corn meal; 30 pounds ground oats; 30 pounds middlings; 13 pounds gluten feed.

Ration 8. One-half corn ration No. 1; one-half wheat ration No. 3.

Ration 9. 90 pounds wheat meal; 10 pounds oil meal.

Ration 10. 30 pounds corn; 30 pounds ground oats; 30 pounds middlings; 10 pounds oil meal.

The experiments were conducted for the most part from spring until fall, making the temperature conditions favorable for most

[11] This *Journal*, iii, p. 307, 1907.

[12] *Bulletin 207, Ohio Experiment Station.*

of the time. The records of growth are recorded in Table I. In addition charts giving the curves of growth are added for a number of the animals.

The normal curve of growth for swine is taken from the results of experiments conducted at American Experiment Stations and

TABLE I.

Record of live weight of swine.

Ration 1 (Corn).

DATE	ANIMAL 1	ANIMAL 2
	lbs.	*lbs.*
August 5.....	40	45
September 30...	45	60
October 28.....	49	64
November 5.....	50	65
December 9.....	50	66
January 5.....	46	61
Discontinued		

Ration 2 (Corn + Salts).

DATE	ANIMAL 3	ANIMAL 4
	lbs.	*lbs.*
March 11.....	49	55
April 22.....	63	72
June 17.....	82	104
July 8.....	89	123
August 26.....	116	165
September 30....	142	195
October 28.....	171	218
November 18.....	188	234
December 16....	222	262
December 30....	235	270

Ration 3 (Wheat).

DATE	ANIMAL 5	ANIMAL 6
September 16...	70	70
September 30...	70	78
October 28.....	87	93
November 18....	100	101
December 16....	107	95
December 30....	104	87
Discontinued		
Pigs stiff and rough coated		

Ration 4 (Wheat + Salts).

DATE	ANIMAL 7	ANIMAL 8
March 11.....	44	53
April 22.....	68	80
June 17.....	82	89
July 8.....	89	97
September 30....	92	110
October 28.....	86	124
November 18..	Discontinued	Ration changed. Grew fat but not tall. In poor condition.
November 18.....		163

TABLE I.—Continued.

Ration 5 (Wheat + Wheat Bran).

DATE	ANIMAL 9	ANIMAL 10
	lbs.	*lbs.*
December 30....	50	31
January 28.....	57	38
February 25.....	61	43
March 4.....	63	43

Discontinued

DATE	ANIMAL 11	ANIMAL 12
August 5.....	44	41
September 30....	57	57
October 28.....	63	61
November 5.....	62	62
December 9.....	61	66
January 5.....	60	56

Discontinued

Ration 6 (5 + Salts).

DATE	ANIMAL 13	ANIMAL 14
	lbs.	*lbs.*
January 7.....	33	33
March 18.....	46	49
April 15.....	55	57
June 3.....	57	60

Discontinued

Ration 7 (Mixed Grain).

DATE	ANIMAL 15	ANIMAL 16
September 16...	60	52
September 30...	70	60
October 28.....	85	76
November 18....	96	90
December 16....	100	90
December 30....	102	87

Pigs stiff
Discontinued

Ration 8 (½ Corn + ½ Wheat).

DATE	ANIMAL 17	ANIMAL 18
September 16....	73	54
September 30....	82	54
October 28.....	89	61
November 18....	108	69
December 16....	114	72
December 30....	114	69

Discontinued

Ration 9 (Wheat + Oil Meal).

DATE	ANIMAL 19	ANIMAL 20
September 16...	63	68
September 30...	70	70
October 28.....	92	85
November 18....	100	94
December 16....	96	95
December 30....	87	96

Discontinued

Ration 10 (Mixed Grain).

DATE	ANIMAL 21	ANIMAL 22
June 17.....	45	45
July 8.....	58	66
September 30....	94	112
October 28.....	100	121
November 18....	102	124
December 16....	100	125
December 30....	92	118

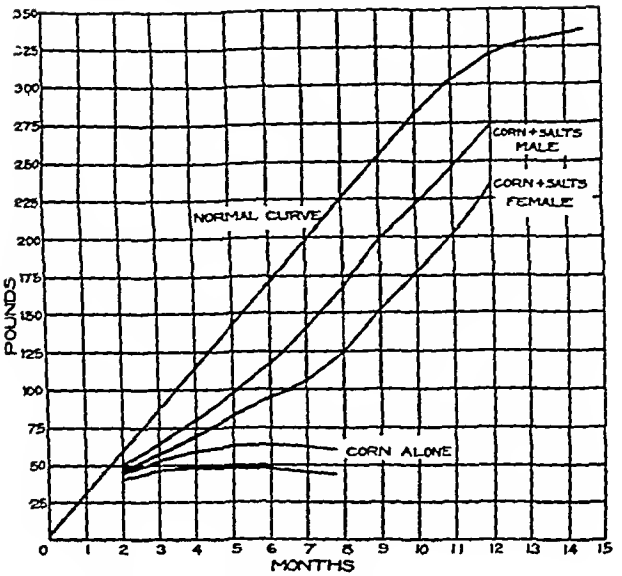


CHART 1. Shows growth of pigs on corn alone and corn plus salts.

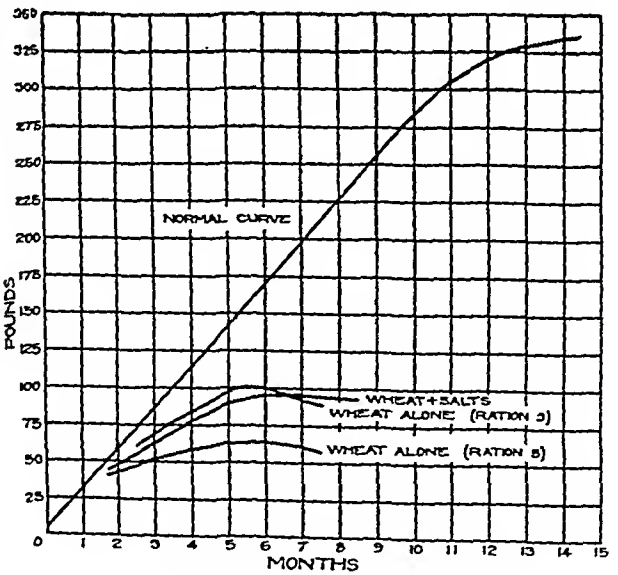


CHART 2. Shows failure of swine to grow on wheat alone or wheat plus salts.

summarized by Henry.[13] This curve is one for pigs confined in pens and consuming a variety of foods and natural water. A daily increase in body weight of 0.9 pound is the average result of these records. In constructing the curve this figure was allowed as the average increase up to about 300 pounds, after which there is a falling off in the daily rate of gain.

It is evident from these records that the corn grain ration alone would not induce growth, but when a suitable salt ration was

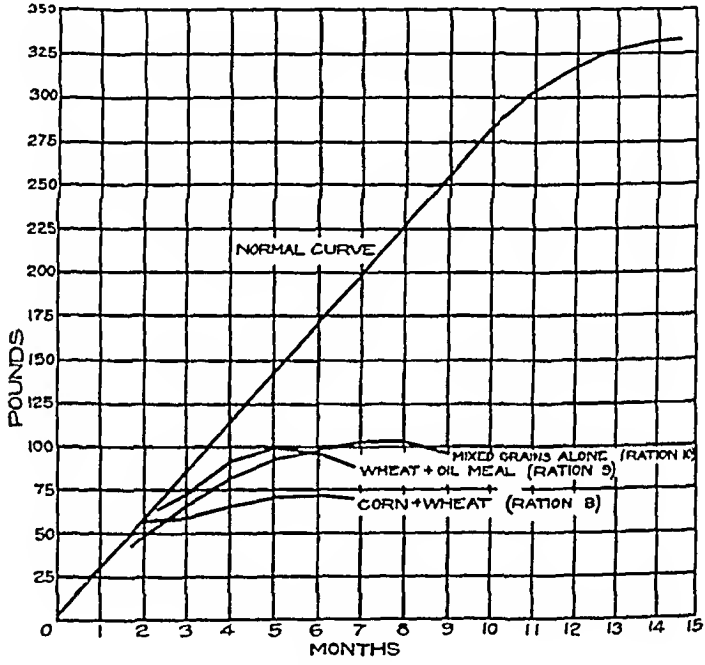


CHART 3. Curves of growth for swine fed mixtures of grains or grain products. Note the failure of continued growth.

added a rate of growth approximating that of the normal curve for pen-confined animals was secured. It is possible, of course, that other salt mixtures might have led to equally good results, but no other mixture was tried with corn. It has been emphasized by Osborne[14] that the probable reason for the failure of swine to grow normally when fed corn alone lies in its large proportion of zein—a protein inadequate for growth when fed alone. Osborne

[13] *Feeds and Feeding*, 7th Ed., p. 549.

[14] *Science*, N.S., xxxvii, p. 185, 1913.

says: "The results here presented leave no doubt that the deficiency observed in the practical feeding of corn grain is explained largely, if not wholly, by the unique chemical constitution of zein which forms such a large part of its proteins." It is apparent from our records that in the natural grain with its mixture of proteins the proteins other than zein must furnish certain quantities of those amino-acids which are missing from zein and which cannot be synthesized by the animal.

In a later publication Osborne and Mendel[15] modified this earlier view and feel less justified in making deductions for the total protein content of corn grain from results secured with zein. It is, however, very probable that fortifying the mixture of corn proteins with a protein like casein would have materially helped the rate of growth.

Those animals receiving the corn-salt ration remained in apparent good health, with sleek coats and no evidence of malnutrition. Those without the salts experienced loss of appetite, stiffness of joints and roughness of coat—evidences of malnutrition. When No. 3, the female in the lot receiving salts, reached a weight of approximately 214 pounds, she was bred. The fetuses were carried through the normal time of gestation, but at the period for parturition there was failure to expel the young. The animal sank into a comatose condition and was killed. From the uterus there were taken eight living young of a total weight of 18 pounds 6 ounces, with individual weights varying from 1 pound 10 ounces to 3 pounds, which agrees well with the weights for animals fed mixed rations. The young were hairless and alive when removed from the mother. No attempt was made to rear any of them. Lack of exercise for the mother is given by Animal Husbandry experts as the cause of this failure to expel the young. More data, of course, are necessary for a safe conclusion that the corn ration plus salts will, in addition to growth, give a complete cycle of life, but it is evident that a very considerable amount of growth can be obtained on the corn grain and its proteins alone, when a proper salt mixture is present.

It is further evident that even with mixed grains without added salts no appreciable and continued growth could be obtained. In

[15] This *Journal*, xviii, p. 1, 1914.

the early history of the experiment there was always some growth which might approximate the normal curve, but in no case was active cell building continued at a normal rate. These results are extremely important, indicating what a large factor in the growth curve must lie in those extraneous conditions usually surrounding the animal, but to which so little heed is given and concerning the details of which we understand so little. The salts carried by natural water or obtained by the animal from the soil are evidently very important factors in promoting growth when the ration is restricted to the grains.

Besides the records here published we have records of swine receiving ration 1, but given natural water and allowed the run of a small paddock free from vegetation. These animals grew normally from weights of 47 and 56 pounds, respectively, to 368 and 385 pounds. They were started in December, but the rate of growth was not as rapid as later, or until early April, when frost left the ground and active rooting could take place. In some instances the rate of growth during a period of active rooting was double that of the winter environment. Other records show that animals receiving a mixture of grains made of one-third corn ration, one-third wheat ration and one-third oat meal, but given natural water and the run of a paddock, grew normally from weights of 47 and 53 pounds, respectively, to 345 and 432 pounds. It is very evident from such results that the salts of the water or possibly unknown substances in the soil or tap water were important factors in differentiating between these results and those secured under confinement and distilled water.

With the wheat ration and no salts, as with the corn ration minus salts, no appreciable and continued growth occurred. Here again loss of appetite, stiffness in the joints and roughened coat followed a few weeks of confinement to this diet. With the addition of a salt mixture, approximating that of a successful artificial rat ration, better growth was secured, but it never approached a normal curve and evidences of malnutrition soon developed. No. 7 failed to grow after reaching 92 pounds, with a gradual decline from that time. No attempt was made to revive this animal. No. 8 continued to grow in weight somewhat beyond that point, finally reaching a weight of 124 pounds, when decline set in and the ration was changed to one of mixed grains,

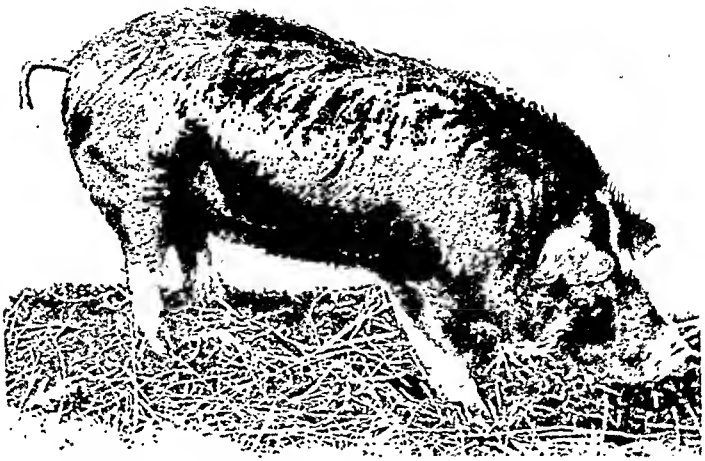


FIG. 1. A WHEAT-SALT MIXTURE FED PIG. Note the lack of thrift in this animal. 90 pounds when photographed. Growth had ceased.

FIG. 2. A CORN-SALT MIXTURE FED PIG. Note the healthy appearance of this animal. 270 pounds when photographed.

milk and natural water. On this ration growth (?) (increase in weight) was resumed. This animal, after the change in ration, became very fat and of abnormal shape. There was little or no expansion in length or height, the animal measuring but 19 inches in height at the front legs, while on the same date those animals receiving corn and salts measured 23 and 26 inches, respectively. Evidently the inherent power for growth had been appreciably suppressed with a stunted pig as a result, but this power had not been entirely lost. In addition to overfatness, dragging of the hind quarters and general paralysis, these animals showed a peculiar deflection of the head, evidently due to an unequal tonus of the muscles of the neck. We record this phenomenon here as it has also been observed in the weak offspring from cows fed on the wheat plant. In one instance, with apparently an exceptionally strong individual, we were able to get considerable growth on the wheat ration (ration 5) plus natural water and the run of an outdoor paddock, but the growth fell below the normal. Other individuals under this same environment made only slight growth.

In these cases of nutrition on the wheat ration we are apparently not dealing wholly with a lack of salts or any other necessary constituents for growth, as evidenced in the experiments which follow and which were carried out in 1913–14, but with some toxic substance either resident in the grain or produced in the animal from wheat constituents. Whether the toxic substance can be destroyed by heat, germination or nullified in its action by anti-substances present in other natural food materials which may accompany wheat in the ration are questions not settled by these experiments. These questions are, however, partly answered in the experiments that follow.

EXPERIMENTS. (*Swine, 1913–14.*)

From what has preceded it was evidently important to repeat certain phases of the work and to eliminate from the rations the possible factor of lack of supply of all constituents now known to be necessary for growth; consequently besides salts the wheat ration was fortified with butter fat in order to cover the possibility of the wheat grain being deficient in those substances now

known to be carried by butter fat and able to promote growth in restricted artificial rations. A further possibility of inadequate proteins was met by adding a quantity of casein to the wheat ration equivalent to 18 per cent of the total protein fed and 2.5 per cent of the ration. Hydrolysis of wheat gluten has been made by Abderhalden,[16] showing the presence of the cyclic compounds tryptophane and tyrosine, as well as all of the diamino-acids, making it extremely probable that the ensemble of wheat grain proteins is not deficient in "building stones" for the animal, although they may not be present in a proportion suitable for most rapid growth.[17] The salt mixture used in these experiments was like that used in the corn ration in the previous year. The rations used were as follows:

Ration 1. 95.5 pounds wheat meal; 2.5 pounds wheat gluten; 2 pounds corn oil; (323 grams K_2HPO_4; 513 grams Ca lactate).

Ration 2. 95.5 pounds wheat meal; 2.5 pounds wheat gluten; 2 pounds butter fat; (323 grams K_2HPO_4; 513 grams Ca lactate).

Ration 3. 95.5 pounds wheat meal; 2.5 pounds casein; 2 pounds butter fat; (323 grams K_2HPO_4; 513 grams Ca lactate).

Ration 4. 94 pounds wheat meal; 6 pounds oil meal; (323 grams K_2HPO_4; 513 grams Ca lactate).

Ration 5. 30 pounds corn; 30 pounds oats; 30 pounds middlings; 10 pounds oil meal; (323 grams K_2HPO_4; 513 grams Ca lactate).

Ration 6. 98 pounds wheat flour; 2 pounds wheat gluten; (323 grams K_2HPO_4; 513 grams Ca lactate).

Distilled water and confinement to pens were strictly followed as in the former year. Such conditions do not apparently restrict growth for this class of animals when the ration is physiologically balanced. The animals were started on the rations at a somewhat greater weight than in the previous year. They were of vigorous, healthy type, of from 70 to 120 pounds, respectively.

The live weight records secured are recorded in Table II which follows. Beyond the table are charts showing the curves of growth for an individual from each group.

The most striking results to be noticed in these experiments are the normal and sustained growth induced by the substitution of casein for the wheat gluten in the ration. It might be urged

[16] *Zeitschr. f. physiol. Chem.*, xlvii, p. 354, 1906.

[17] McCollum: this *Journal*, xix, p. 323, 1914.

TABLE II.

Records of live weight of swine.

Ration 1 (Wheat, Corn Oil, Salts).

DATE	ANIMAL 1	ANIMAL 2
	lbs.	*lbs.*
April 7	115	118
May 5	137	149
June 2	128	154
June 30	130	159
July 28	(out)	164
August 11		164

Ration 2 (Wheat, Butter Fat, Salts).

DATE	ANIMAL 3	ANIMAL 4
	lbs.	*lbs.*
April 7	88	86
May 5	106	112
June 2	115	124
June 30	125	142
July 28	134	154
August 25	151	182

Ration 3 (Wheat, Casein, Butter Fat, Salts).

DATE	ANIMAL 5	ANIMAL 6
April 14	108	98
May 12	145	124
June 9	173	144
July 7	205	175
August 4	219	207
September 1	250	223

Ration 4 (Wheat, Oil Meal, Salts).

DATE	ANIMAL 7	ANIMAL 8
April 7	113	99
May 5	150	135
June 2	158	150
June 30	167	161
July 28	162	165
August 11	160	166

Ration 5 (Mixed Grain, Salts).

DATE	ANIMAL 9	ANIMAL 10
April 7	80	80
May 5	112	94
June 2	120	108
June 30	126	118
July 28	119	122
August 25	121	125

Ration 6 (Wheat Flour, Salts).

DATE	ANIMAL 11	ANIMAL 12
April 7	110	123
May 5	117	156
June 2	115	156
June 30	112	146

that this added casein served solely as a source of needed amino-acids which the wheat proteins could not furnish in adequate quantities for a rapid expansion of cells. This may have been the primal effect, but there certainly must have been secondary influences accompanying rapid growth. There is undoubtedly very great significance in the idea of a suitable proportion of amino-

acids in the ration, yet a pig fed wheat, wheat gluten and salt is not an animal suffering from starvation; but the paralysis, blindness, roughness of coat, and general debility accompanying a long continued use of this ration are all indicative of an inherent toxicity in the wheat kernel. Supplementing with casein does, very probably, make available a more suitable mixture of amino-acids which then makes possible a rapid tissue growth; but

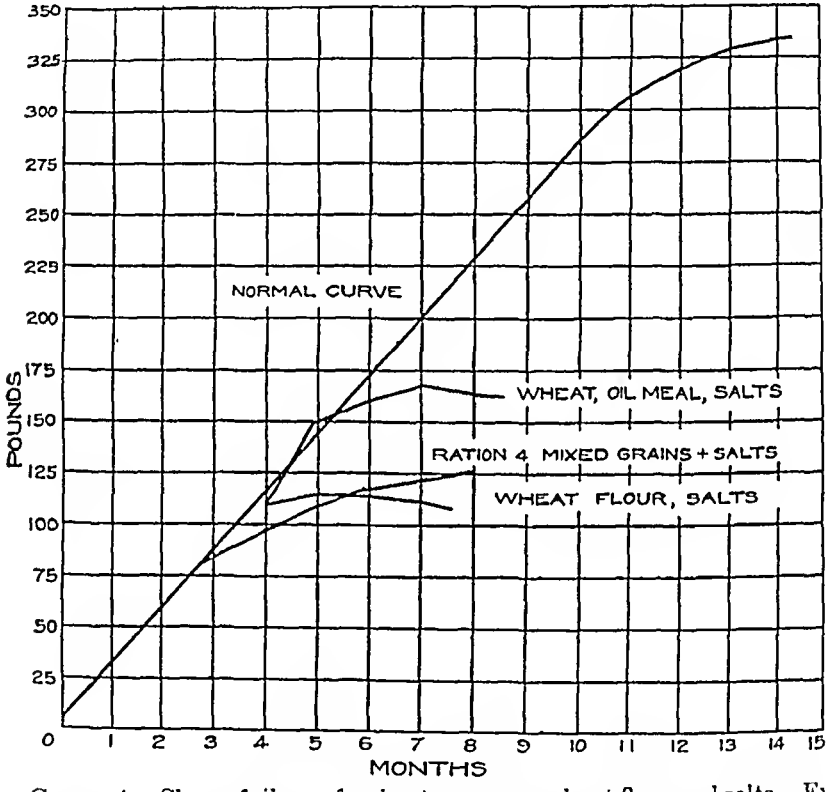


CHART 4. Shows failure of swine to grow on wheat flour and salts. Even on mixed grains and salts a normal curve of growth was not obtained (ration 5 and not 4 as stated in chart). There was improvement when oil meal supplemented wheat, but a normal growth was not produced.

this rapid cell expansion is really, in our judgment, an important factor in counteracting the depressing influence that follows the use of wheat in large proportions in the ration. Satisfying the strong impetus to growth has increased, in a very large measure, the resisting powers of the animal. The results have a bearing, not only on protein supplements as sources of needed amino-

acids in a natural diet, but on the relation this may bear to the restorative factors that may be made operative in certain conditions of malnutrition.

We would not insist that the toxicity of wheat rests in the presence of a substance which finds no place in normal biological processes. The decided improvement in nutrition which results from the addition of casein and salts would seem, in our judgment,

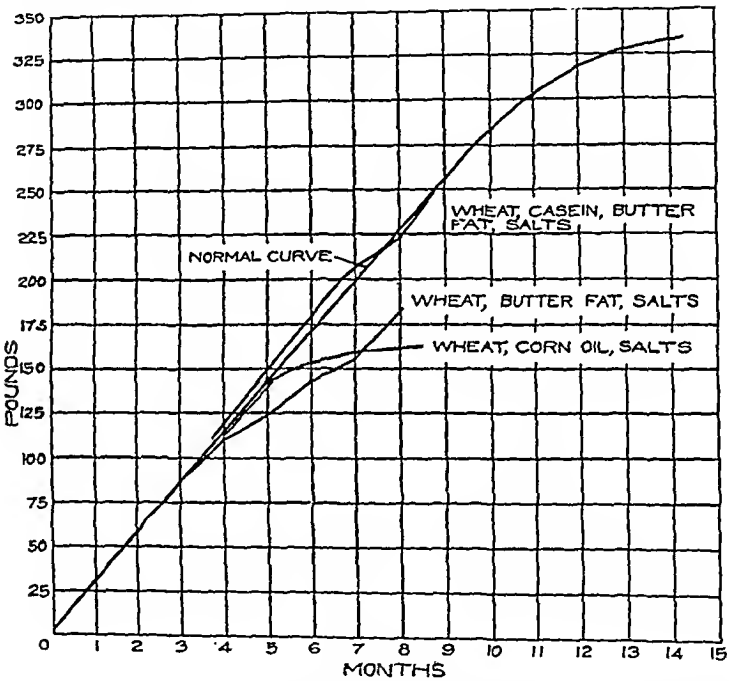


CHART 5. These curves show the marked improvement of wheat for growth when supplemented with butter fat and salts and especially when supplemented with casein, butter fat and salts. Corn oil and salts were not effective for continued growth.

to suggest that the explanation may rest in the presence in the wheat proteins of excessive amounts of certain amino-acids which under the peculiar circumstances attending the taking of this restricted ration injure the cell.

It is evident that wheat proteins cannot be efficiently converted into metabolizing tissue, yet the cells of the tissues must be

saturated with an ill balanced mixture of amino-acids when wheat alone is fed. Lusk[18] has shown that the "specific dynamic action" of proteins rests in the pronounced pharmacological action of certain amino-acids. We would ask: May not the presence of an incomplete mixture of amino-acids in the body fluids in relatively high concentration and uniformly maintained for a long period exert a toxic action on the cells? As soon as the cleavage products of the wheat proteins are supplemented by those of casein, a mixture much more suitable for growth results, with a corresponding decrease in the amount of the residual fraction which cannot be converted into metabolizing tissue. This would account for the phenomena recorded in our experiments.

It is clear that this idea is one which can be readily tested by experimental methods, and an inquiry into its validity will be made.

Where butter fat was added to the wheat-salt ration very great improvement in the rate of growth was secured, but not quite a normal curve of growth was produced, as was the case when both butter fat and casein were added. These animals remained in prime condition for about five months, with no evidences of malnutrition appearing. At the end of this time, however, one of the individuals became very stiff and symptoms of malnutrition appeared. The improvement made by the addition of butter fat alone is in marked contrast to the effect secured where corn oil was added. In the latter case very little growth could be secured after feeding for thirty days, although there was maintenance for a much longer time. These animals, however, became stiff, the coats rough and there was an apparent lack of thrift and physiological balance. Much the same condition obtained with those receiving the added oil meal. There was a rapid early growth on these rations, but the curve soon dropped to one of maintenance.

With animals receiving flour and salts very little growth was secured and loss of appetite and weakened condition manifested itself very early. Partial blindness in one of the individuals appeared at the end of two months' feeding. In addition there was a loss of muscular coördination and a marked scurfy condition of the skin.

[18] Lusk: this *Journal*, xiii, pp. 155 and 185, 1912.

It is evident that the whole wheat grain or wheat flour with or without salts will not sustain growth and even leads to physiological disturbances when continued as the sole source of the nutrients for but a short span of the entire period of growth of this species.

When, however, there was fortification with butter fat or, better, with butter fat and casein, a healthy, vigorous animal could be secured, although the normal rate of growth was not secured with the fat alone.

Fortifying the wheat kernel with oil meal (a concentrated mixture of plant proteins) or with corn oil improves it but slightly for sustained growth.

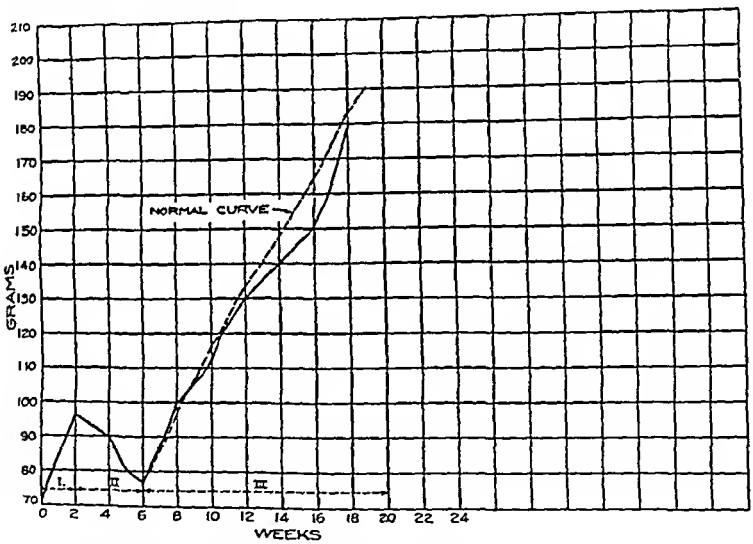


CHART 6 (Female). Showing normal growth on wheat with 15 per cent of milk powder (Period I), and prompt decline when the ration was changed to starch, wheat gluten and a salt mixture (Period II). There was a prompt resumption of growth at about normal rate when the ration was changed to a mixture of casein, dextrine, lactose and salts (Period III). A large part of the ration can be derived from wheat without interference with normal growth.

Salt Mixture.

	grams		*grams*
Calcium lactate	0.785	$CaH_4(PO_4)_2H_2O$	0.873
K_2HPO_4	1.336	Mg citrate	2.489
$MgSO_4$ (Anhyd.)	0 069	Na citrate (Anhyd.)	0.048
NaCl	0.030		

EXPERIMENTS. (*Rats.*)

The experiments with rats illustrated by charts 6–11 show that this species is affected in a manner similar to swine when confined to a ration of wheat alone. Correction of the mineral content of the ration alone (chart 11) induces a certain amount

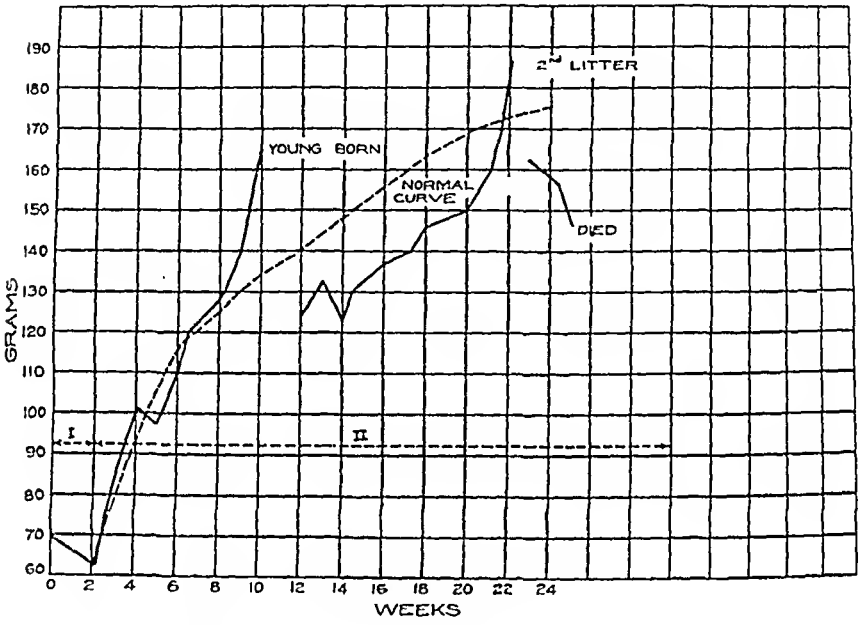


CHART 7 (Female). This curve illustrates the failure of the ether extract of egg yolk to supplement wheat for the nutrition of the rat (Period I). When the ration consisted of wheat 80 per cent and dry egg yolk 20 per cent, normal nutrition with the production of two litters of young was secured (Period II).

The rations employed were:

Period I.	*per cent*	*Period II.*	*per cent*
Wheat	95	Wheat	80
Ether extract of egg yolk	5	Dry egg yolk	20

of growth, but only temporary benefit is secured by this means. Calcium lactate alone will induce growth for a short time. Similarly the fats of the egg (chart 7) fail to supplement wheat and induce physiological well-being.

Sprouting the wheat exerts a marked influence on the animals as indicated by lengthening of life. When the wheat is sprouted

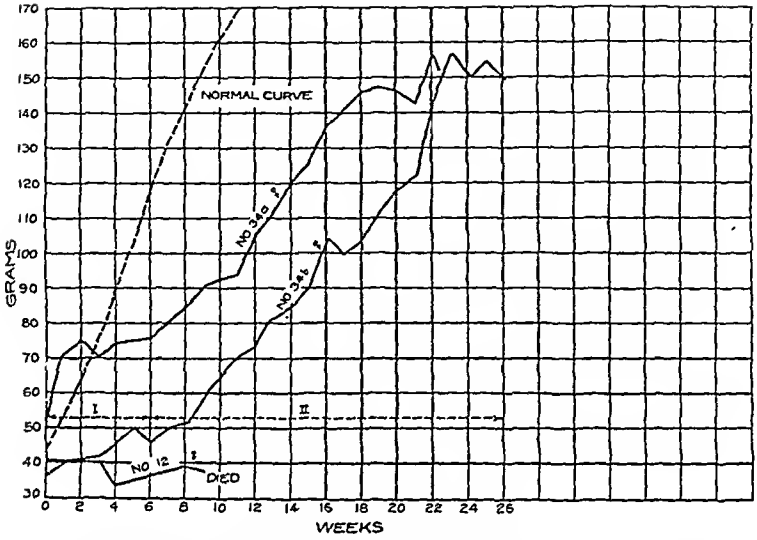


CHART 8 (Males). *Period I.* The lower curve shows the typical behavior of rats fed wheat alone with distilled water. No growth is secured and the animals rarely survive beyond sixty days. The upper curves illustrate the effect of the addition of calcium lactate to wheat. This induces a small amount of growth for a short time, but has only a very temporary beneficial effect.

Period II. Illustrates the marked influence on growth of supplementing wheat with casein and a suitable salt mixture.

The rations employed were as follows:

Period I.

	grams
Wheat	100.00
Calcium lactate	1.58

Period II.

	grams
Wheat	90
Casein	10
Salt mixture	3

The salt mixture had the following composition:

	gram		gram
NaCl	0.146	K_2HPO_4	0.805
$MgSO_4$ (Anhyd.)	0.225	$CaH_4(PO_4)_2H_2O$	0.456
$NaH_2PO_4H_2O$	0.253	Fe lactate	0.100

and calcium lactate is added, a very slow but long sustained growth was observed (chart 9). The addition of butter fat to sprouted wheat with calcium lactate did not appreciably better the condition of the animals.

Charts 6, 7 and 10 illustrate the fact that 80 to 90 per cent of the ration may consist of wheat without any bad effects when

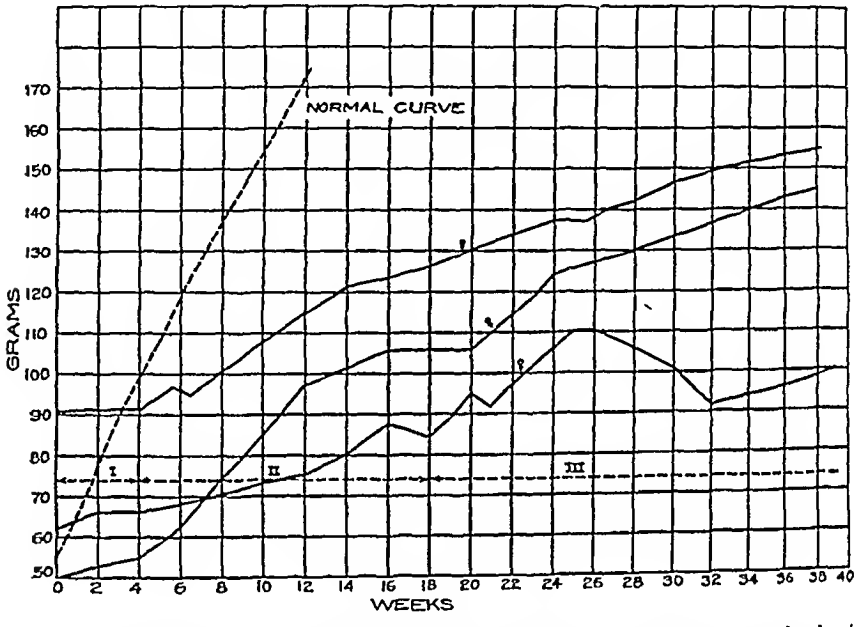


CHART 9 (Males). Throughout the experiment freshly sprouted wheat was furnished the animals daily. No growth was secured. In Period II calcium lactate (1.58 grams per 100 wheat) was added to the sprouted wheat, and growth at a slow rate resulted during 85 days. In Period III both calcium lactate and butter fat (5 per cent) were added. The prolongation of life and great improvement in well-being which results from sprouting the wheat and the addition of calcium lactate is easily seen.

the ration is supplemented with milk or egg yolk. Chart 8 shows how markedly the well-being of rats is improved by the addition of casein and correction of the mineral content of wheat by a suitable salt mixture.

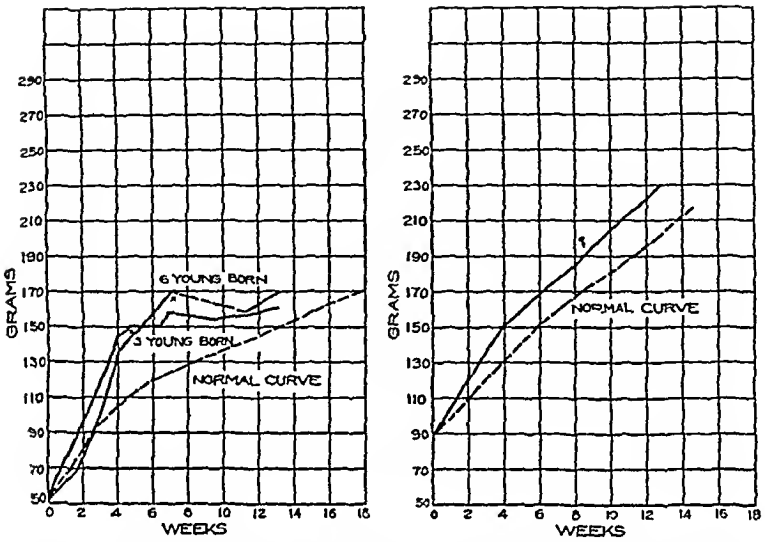


CHART 10. Illustrates the attainment of normal growth and reproduction on a ration containing 64 per cent wheat, supplemented with milk powder (10 per cent) and casein (10 per cent), butter fat (5 per cent) and a salt mixture.

The litter of three young weighed, when 16 days old, 56 grams. When 33 days old they weighed 190 grams. Two of the young, which were continued on the mother's ration, weighed when 45 days old, 100 and 110 grams, respectively.

The six young in the other litter weighed, collectively, 248 grams when 23 days old. These weights are normal for young produced on mixed rations.

Ration employed.

	grams
Milk powder	10
Casein	10
Wheat	64
Dextrine	7.4
Butter fat	5.0
Salt mixture	3.68

Composition of salt mixture.

	grams
NaCl	0.520
$CaH_4(PO_4)_2H_2O$	0.276
Ca lactate	1.971
K citrate	0.799
Fe acetate	0.100

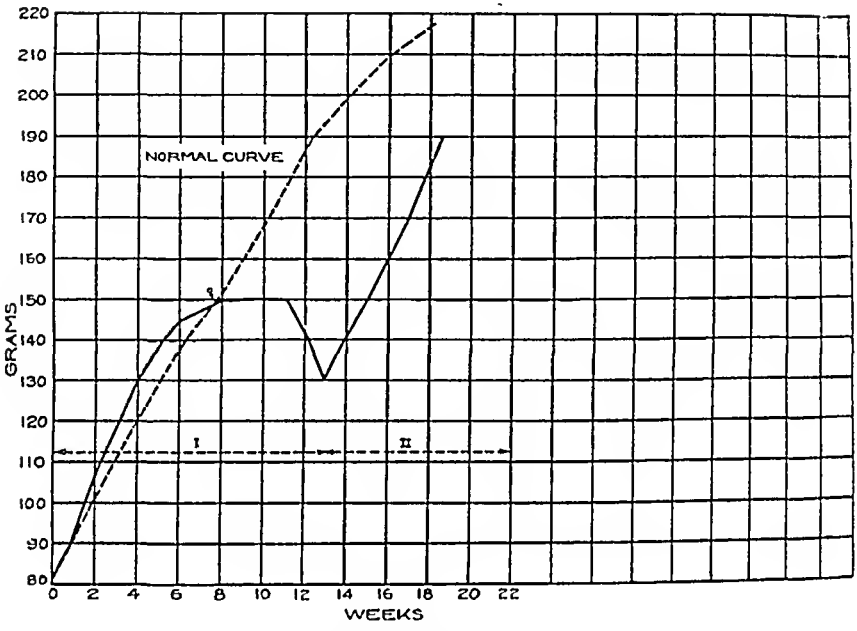


CHART 11 (Male). Illustrates the effect of supplementing a mixture of wheat and wheat gluten with lard and a suitable salt mixture. For a time rapid growth was secured, but after two months failure of nutrition ensued (Period I). Period II shows the prompt recovery on a mixed ration of naturally occurring foodstuffs.

Ration.

	grams
Wheat	50
Wheat gluten	17
Lard	12
Starch	10
Salt mixture	10.8

Salt mixture employed.

	grams
$CaH_4(PO_4)_2H_2O$	2.15
Ca lactate	4.51
$MgSO_4$ (Anhyd.)	0.06
NaCl	1.35
Na_2SO_4	0.72
K citrate	2.69

SUMMARY.

1. When swine are restricted to corn meal and gluten feed little or no growth can be secured, but with an addition of salts, making the entire ash content of the ration very similar in quality to that of milk, growth approximating that of a normal curve was secured to at least 275 pounds. These results are not in harmony with the theory that the failure of swine to grow on corn alone is due entirely to the incomplete nature of its protein content.

2. Restriction to mixed grains and distilled water did not allow normal growth with swine. This emphasizes again the very great importance of either the mineral side of a ration, or as yet unknown factors operative in the normal environment of this species, namely, soil rooting, natural water, etc.

3. When the wheat kernel supplied all the nutrients, growth was again limited with both swine and rats. However, when the salt content was modified to resemble milk some growth could be secured, but ultimately this ceased, followed by partial paralysis, particularly in swine, and a general decline. Correcting the mineral content of the wheat kernel alone induces a certain amount of growth, but the benefit is only temporary.

4. When the wheat kernel was fortified with salts and butter fat, the growth curve was very much improved in both species, although a normal curve was not secured. The animals, however, remained vigorous and strong for a very much longer period, although partial decline in some individuals, mainly characterized by stiffness, ultimately set in. However, when the wheat kernel, salt and butter fat ration was supplemented with casein to the extent of 2.5 per cent of the ration, a normal curve of growth was secured for swine. Similar results were secured with rats.

5. Rations may contain as much as 80 to 90 per cent of wheat without bad effects when supplemented with milk or egg yolk. Normal reproduction as well as normal growth have been secured with such rations.

THE ACTION OF RENNIN ON CASEIN.

By ALFRED W. BOSWORTH.

(*From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y.*)

(Received for publication, September 22, 1914.)

In order to determine if the change from casein to paracasein results in the cleavage of any of the elements contained in the casein molecule, it is imperative that *pure* casein be used as a standard of comparison, and that the rennin activity be positively differentiated from any further proteolytic activity of the enzyme under consideration; for it is quite evident that "Rennin action is probably a hydrolytic cleavage and may be considered the first step in the proteolysis of casein. It would follow from this that the action now attributed to rennin may be produced by any proteolytic enzyme."[1]

Pure casein and paracasein have been prepared according to the methods previously published.[2] Pure paracasein was also prepared by allowing trypsin to act upon fat-free milk, after the addition of calcium chloride, and the curd produced was purified according to the method referred to. The use of an excess of ammonia as prescribed has been criticized by Harden and Macallum,[3] who claim that preparations made in that way may have a low phosphorus content due to the cleavage of phosphorus from the casein molecule by the action of the ammonia. In a recent paper[4] it has been shown that this criticism does not hold. The analyses of the preparations are given in the table.

These figures show that the composition of paracasein is the same irrespective of the enzyme used to produce it. The figures also show that casein and paracasein have the same percentage composition which excludes the possibility that cleavage of any of the elements of casein is a result of its transformation into paracasein by enzymes.

[1] Bosworth: this *Journal*, xv, p. 236, 1913.
[2] Van Slyke and Bosworth: *ibid.*, xiv, p. 203, 1913.
[3] Harden and Macallum: *Biochem. Journ.*, viii, p. 90, 1914.
[4] Bosworth and Van Slyke: this *Journal*, xix, p. 67, 1914.

	CASEIN	PARACASEIN BY RENNIN	PARACASEIN BY TRYPSIN
Moisture	1.09	1.63	1.27
Carbon in dry substance	53.50	53.50	53.47
Hydrogen in dry substance	7.13	7.26	7.19
Oxygen in dry substance	22.08*	21.94*	22.04*
Nitrogen in dry substance	15.80	15.80	15.78
Phosphorus in dry substance	0.71	0.71	0.71
Sulphur in dry substance	0.72	0.72	0.72
Ash in dry substance	0.06	0.07	0.09

* By difference.

Harden and Macallum, in their paper, conclude that "The conversion of caseinogen into casein by enzyme action is accompanied by the cleavage of nitrogen, phosphorus and calcium."[5] It seems more probable to us that this cleavage follows, rather than accompanies, the conversion in question, and is to be attributed to a continuation of proteolytic activity by the enzyme beyond the point where casein has been changed to paracasein. This point was emphasized in my first paper on the action of rennin on casein.[6]

The similarity between the composition of casein and paracasein, and the fact that casein has been shown to have a molecular weight of 8888 ± and a valency of 8, while paracasein has been shown to have a molecular weight of 4444 ± and a valency of 4,[7] seems to be evidence enough for concluding that the transformation of casein into paracasein is a process of hydrolytic splitting, one molecule of casein yielding two molecules of paracasein, and that this splitting of casein is not accompanied by a cleavage of any of the elements contained in the original casein molecule.

[5] The English caseinogen is equivalent to the American casein.

The English casein is equivalent to the American paracasein.

Harden and Macallum give the nitrogen-phosphorus ratio of casein as N: P = 100: 5.6. The high phosphorus content of their casein preparations (0.87–0.90 per cent) would seem to indicate the presence of considerable inorganic phosphorus. If our figures are correct for the nitrogen and phosphorus content of casein (15.80 per cent N and 0.71 per cent P), the ratio would be N: P = 100: 4.5. In only one of their experiments conducted to show the loss of phosphorus from the casein molecule was this ratio reduced to 4.5.

[6] Bosworth: *ibid.*, xv, p. 231, 1913.

[7] Van Slyke and Bosworth: *ibid.*, xiv, p. 227, 1913.

ACIDOSIS IN OMNIVORA AND HERBIVORA AND ITS RELATION TO PROTEIN STORAGE.[1]

By H. STEENBOCK, V. E. NELSON and E. B. HART.

(From the Department of Agricultural Chemistry of the University of Wisconsin.)

(Received for publication, September 24, 1914.)

The opinion is commonly held and now fully established by experimental work[2] that omnivora and carnivora can effectively protect themselves against acidosis (excess of acids over bases) in the tissues by ammonia production, but that herbivora have no such power, or at least only to a limited extent. This latter view rests primarily upon early records secured with rabbits. However, in 1898 appeared the important work of Winterberg[3] which showed conclusively that rabbits fed on oats could protect themselves against ingested mineral acids by coupling these with ammonia and excreting the ammonium salts through the urine. Accompanying this utilization of ammonia for purposes of neutralization there was a reduction in the output of urea. This work established the principle that there was no difference in the ability of carnivora or herbivora to maintain tissue neutrality through the production or the utilization of the excess of ammonia produced in the organism. In 1904 Baer,[4] from his work on the goat, pig and monkey, showed that these animals under conditions of starvation increased the output of urinary ammonia, and concluded that very probably this ammonia originated in the tissues for the purpose of maintaining neutrality. It has further been established by McCollum and Hoagland[5] that an omnivorous animal (the pig) is able to maintain tissue neutrality, or at least raise the ammonia production on a nitrogen-free diet plus mineral

[1] Published by permission of the Director of the Agricultural Experiment Station.

[2] Walter: *Arch. f. exp. Path. u. Pharm.*, vii, p. 148, 1877.

[3] *Zeitschr. f. physiol. Chem.*, xxv, p. 202, 1898.

[4] *Arch. f. exp. Path. u. Pharm.*, liv, p. 153, 1906.

[5] This *Journal*, xvi, p. 299, 1913-14.

acids. Such a diet results in an increased ammonia production in the urine with decreased urea output and increased total nitrogen excretion. Apparently then, even with the exogenous nitrogen factor excluded, ammonia production will rise and fall, depending upon the balance of base and acid radicals in the ration or tissues of the animal.

Of late, considerable emphasis has again been placed upon the balance of acid and base forming elements in rations or diets with the constant emphasis placed upon the necessity of maintaining an excess of basic over acid radicals for normal nutrition (Forbes,[6] Sherman and Gettler,[7] Kastle[8]). Apparently in this emphasis of a balance of acid and base forming elements there is forgotten the part ammonia can play in maintaining tissue neutrality and the abundance of ammonia available for such purposes on the ordinary diet. Ammonia production in the animal organism is under certain conditions probably quantitatively determined by the necessity for forming salts. However, the choice of an alkaline diet may have its wisdom in more complicated and obscure factors than maintaining neutrality itself, important as this is; but experimental evidence on this phase of the question is not extensive. We refer to the question of ease of solubility of cellular by-products in acid or alkaline media, combination of carbon dioxide in the blood, etc.

Again, a moderate acidity such as may occur in natural foods may be very much less dangerous than an imposed mineral acidity with its possible attendant harm due to the production of an excess of ammonium salts in the tissue and a combination of the acid in fixed and stable salts with other tissue bases. In fact the question of the effect of a natural acid food will not be settled until animals have been maintained on such materials through the cycle of life and a sufficient number of animals involved, both weak and strong, so as to eliminate the factor of individual resistance which may be shown by the more vigorous and hardy.

Since swine are more liable than any other animal to be fed exclusively grain rations, which are always acid, our first experi-

[6] *Bulletin 207, Ohio Experiment Station.*

[7] This *Journal*, ix, p. 323. This article gives a complete bibliography of the subject and will not be repeated here.

[8] *Amer. Journ. of Physiol.*, xxii, p. 284, 1908.

ments deal with the rise and fall of urinary ammonia production when this species, as representative of omnivora, is confined to grains alone or grains supplemented with basic materials. Experiments detailed later in the paper deal with the influence of acid rations on ammonia production in herbivora (calves).

EXPERIMENTAL PART. (*Swine.*)

Upon theoretical grounds cereal grain rations are invariably acid. When hydrolized and oxidized in the animal there is a preponderance of acid forming elements over the bases present. Calculations made by Forbes[9] show in certain cereal grains or their by-products an excess of basic elements, but this is due to the use of the percentage of sulphur in the ash of these materials rather than the total sulphur, as would have been done had such data been available. If the total sulphur is considered in the calculation, then in the cereal grains or their by-products there is a preponderance of acid over base forming elements. On such rations one would expect to find the ammonia content of the urine high.

That this is not only theoretically, but also experimentally, true is shown in the following data concerning swine kept upon rations made wholly from the cereal grains, or such grains supplemented by basic salts or such basic natural feeding materials as alfalfa hay. The records given are not from a single animal carried through the different rations, but from an individual in the lot which had received the ration stated for a considerable number of weeks.

Quantitative collection of the urine and daily analysis for total nitrogen, ammonia by Folin's method, acidity to litmus and $\frac{N}{10}$ NaOH were carried out.

On the cereal grain rations the ammonia nitrogen content of the urine was very uniform and approximated 12 per cent of the total urinary nitrogen, but was markedly decreased by the addition of basic materials to the ration.

On all the cereal rations the urine was distinctly acid to litmus and also had a high titration factor to phenolphthalein. On the supplemented rations it became neutral, but rarely alkaline to

[9] *Loc. cit.*

TABLE 1.

Record of reaction of urine, ammonia production, etc. (Swine).

DATE	VOL. OF URINE	REACTION TO LITMUS	TOTAL ACIDITY cc. $\frac{N}{10}$ NaOH	DAILY INTAKE N	DAILY URINARY N	URINARY AMMONIA N	AMMONIA PER CENT OF TOTAL URINARY N
Animal No. 1, 270 lbs.	*Ration: 70 lbs. corn meal, 30 lbs. gluten feed, tap water.*						
	cc.			grams	grams	grams	
October 23	4100	Acid	900.0	37.37	27.75	3.08	11.09
October 24	3350	Acid	752.0	37.37	24.14	2.66	11.01
October 25	4325	Acid	900.0	37.37	32.80	4.22	12.86
October 26	5000	Acid	600.0	37.37	34.97	4.13	11.81
October 27	5650	Acid	840.0	37.37	33.22	4.41	13.27
October 28	4375	Acid	555.0	37.37	29.30	3.69	12.59
Animal No. 2, 270 lbs.	*Ration: 88 lbs. wheat meal, 10 lbs. wheat bran, 2 lbs. wheat gluten, tap water.*						
October 29	4875	Acid	648.0	43.03	29.83	3.65	12.23
October 30	4285	Acid	650.0	43.03	32.80	4.06	12.37
October 31	4975	Acid	850.0	43.03	37.26	4.81	12.90
November 1	4500	Acid	702.0	43.03	31.92	4.61	14.44
November 2	5075	Acid	656.0	43.03	24.18	2.84	11.74
November 3	3150	Acid	1247.0	43.03	21.06	2.67	12.67
November 4	4275	Acid	3163.0	43.03	34.94	4.09	11.70
Animal No. 3, 251 lbs.	*Ration: 70 lbs. oat meal, 30 lbs. whole oats, tap water.*						
November 6	6075	Acid	984.0	41.22	42.41	5.17	12.19
November 7	6300	Acid	743.0	41.22	38.90	4.84	12.44
November 8	4750	Acid	931.0	41.22	38.62	4.46	11.54
November 9	4600	Acid	1012.0	41.22	39.01	4.52	11.58
November 10	4600	Acid	920.0	41.22	34.11	4.04	11.87
November 11	5060	Acid	981.0	41.22	30.41	4.04	13.28
Animal No. 4, 140 lbs.	*Ration: 60 lbs. corn meal, 20 lbs. gluten feed, 20 lbs. alfalfa hay, tap water.*						
January 2	2525	Neutral	181.8	29.10	18.47	1.51	8.17
January 3	3125	Neutral	250.0	29.10	23.74	1.57	6.63
January 4	3625	Neutral	166.7	29.10	23.78	1.12	4.71
January 5	2250	Neutral	58.5	29.10	16.65	0.62	3.73
January 6	3100	Neutral	210.8	29.10	18.68	0.67	3.60
January 7	1769	Alkaline	125.2	38.80	17.13	0.76	4.43
January 8	2618	Alkaline	102.9	38.80	21.98	0.70	3.20

TABLE I—Continued.

Animal No. 5, 222 lbs. Ration: 70 lbs. corn meal, 30 lbs. gluten feed, 276 grams Ca lactate, 338 grams K citrate, 317 grams Mg citrate, salts calculated as quantity necessary to neutralize acidity of ration, tap water.

DATE	VOL. OF URINE	REACTION TO LITMUS	TOTAL ACIDITY cc. $\frac{N}{10}$ NaOH	DAILY INTAKE N	DAILY URINARY N	URINARY AMMONIA N	AMMONIA N PER CENT OF TOTAL URINARY N
	cc.			grams	grams	grams	
February 20	1868	Neutral	219.0	57.40	27.50	2.04	7.41
February 21	1302	Neutral	378.0	15.60	24.30	1.58	6.38
February 22	2052	Neutral	172.0	31.30	31.30	1.68	5.29
February 23	2095	Neutral	147.0	31.30	30.10	2.16	6.80
February 24	1392	Neutral	113.0	31.30	23.70	1.32	5.55
February 25	1741	Neutral	235.0	31.30	24.20	1.27	5.27
Animal No. 6, 252 lbs. Ration: 70 lbs. corn meal, 30 lbs. gluten feed, 2 lbs. wood ashes, tap water.							
March 12	1642	Neutral	32.0	47.04	17.76	0.41	2.33
March 13	1479	Neutral	28.0	31.36	18.36	0.19	1.04
March 14	2520	Neutral	247.0	31.36	24.94	0.31	1.25
March 15	2024	Neutral	39.0	31.36	20.76	0.13	0.67
March 16	1557	Neutral	488.0	31.36	14.28	0.11	0.80
March 17	2300	Neutral	22.0	31.36	18.82	0.15	0.81
March 18	2034	Neutral	51.0	31.36	22.93	0.25	1.09
March 19	1585	Alkaline	46.0	31.36	13.88	0.21	1.53
March 20	2151	Neutral	211.0	31.36	21.86	0.31	1.43

litmus, with a marked decrease in the titration factor, but nevertheless was always acid to phenolphthalein. Where the carbonates were added, urinary acidity to phenolphthalein reached its lowest point, but never entirely disappeared.

The ammonia produced in the urine was 12 per cent of the total urinary nitrogen on the cereal rations and was lowest when carbonates were added, falling on some days to less than 1 per cent, but never entirely disappearing. This rise and fall of ammonia excretion in the urine is to be correlated directly with the reaction of the ration and is now generally recognized as a protective measure against acidosis in the animal. The question of the influence in the tissues of large quantities of ammonium salts on growth and metabolism is not so clear from the data presented,

but must be carefully considered in relation to the efficiency of acid rations. In fact this becomes the real problem in the acid ration for it is now evident that the acidity in this species can be controlled either directly by ammonia production in the tissues, as in endogenous nitrogen metabolism, or by the utilization of that already formed in protein catabolism where the exogenous factor is a large one. The intestine may serve as the main source of the ammonia under conditions of acidosis and where the protein intake is the quantity incident to a normal ration. The result of this ammonia production is a reduction in the output of urea. The influence of ammonium salts on nitrogen retention will be considered later.

EXPERIMENTS WITH HERBIVORA. (*Calves.*)

Exogenous nitrogen metabolism.

In these experiments young calves of different weights were used, with whole milk as the sole source of the nutrients. Milk, on the basis of its acid and base content has a basicity per thousand grams of dry matter equivalent to approximately 175 cc. of normal solution.[10] It was found to be a very simple matter to increase the acidity of this material with a mineral acid and at the same time secure ready consumption. This acidity was controlled by the use of N HCl at various planes. Analysis of the urine for total nitrogen, ammonia, urea and titratable acidity was made. The neutral diet was secured with 10.3 cc. of N HCl per pound of milk. The amount of milk consumed varied with the size of the animal and in some cases reached twenty pounds daily. Before the first records were taken there was a preliminary feeding period, but between the periods where there was a progressive increase in acid ingested there was in some instances no preliminary period in the record. Where the feces were quantitatively collected, a bag as the receptacle for the solid excreta was employed. The records include in some experiments a complete nitrogen balance.

As observed by others in the case of the dog and by us with the pig, under conditions of exogenous nitrogen metabolism with a fairly high plane of protein intake, there was a marked increase

[10] Forbes: *Bulletin 207, Ohio Experiment Station.* Corrected for total sulphur from 0.57 to 2.48 per cent.

TABLE II.

Record of reaction of urine, ammonia production, etc.
Animal No. 1. Calf, 75 lbs.

DATE	VOL. OF URINE	REACTION TO LITMUS	TOTAL ACIDITY cc. $\frac{N}{10}$ NaOH	INTAKE N	URINARY N	URINARY AMMONIA N	AMMONIA N AS PER CENT OF TOTAL URINARY N
12 lbs. whole milk.							
	cc.			*grams*	*grams*	*grams*	
May 18	5346	Neutral	320	25.05	6.88	0.63	9.24
May 19	5423	Neutral	433	25.05	7.59	0.68	8.99
May 20	6089	Neutral	486	25.05	9.49	0.59	6.29
May 21	5185	Neutral	518	25.05	9.64	0.48	5.07
May 22	5007	Neutral	550	25.05	9.74	0.63	6.57
May 23	5686	Neutral	568	25.05	11.24	0.71	6.36
May 24	7035	Neutral	562	25.05	16.43	0.73	4.48
					10.14		6.71
Whole milk + 10.8 cc. N HCl per lb. milk.							
May 28	3808	Acid		25.05	7.09	0.93	13.13
May 29	5094	Acid	1323	25.05	13.76	1.65	12.01
May 30	5409	Acid	1243	25.05	12.86	1.68	13.06
May 31	4994	Acid	1147	25.05	12.02	1.88	15.65
June 1	6082	Acid	1337	25.05	12.56	2.21	17.61
June 2	6089	Acid	1521	25.05	14.91	2.19	14.98
June 3	4911	Acid	1178	25.05	18.56	2.48	13.45
					13.10		14.27
Whole milk + 16 cc. N HCl per lb. milk.							
June 5	4372	Acid	1267	25.05	13.95	2.96	21.26
June 6	4695	Acid	1126	25.05	12.48	2.76	22.11
June 7	4947	Neutral	1384	25.05	16.62	6.45	38.84
June 8	6020	Neutral	1324	25.05	17.02	4.28	25.20
June 9	5695	Acid	1651	25.05	15.94	3.97	24.27
June 10	4620	Acid	1570	25.05	19.40	4.22	21.75
					15.90		25.57

TABLE II—Continued.

DATE	VOL. OF URINE	REACTION TO LITMUS	TOTAL ACIDITY cc. $\frac{N}{10}$ NaOH	INTAKE N	URINARY N	URINARY AMMONIA N	AMMONIA N AS PER CENT OF TOTAL URINARY N
Whole milk + 21 cc. N HCl per lb. milk.							
	cc.			grams	grams	grams	
June 11	7371	Acid	1621	25.05	22.90	4.98	21.77
June 12	5803	Acid	1334	25.05	21.61	5.31	24.59
June 13	5197	Acid	1143	25.05	18.62	3.48	18.69
June 14	5147	Acid	1595	25.05	20.03	3.93	19.66
June 15	5362	Neutral	1158	25.05	20.11	5.64	28.07
June 16	4524	Acid	1583	25.05	18.68	3.10	16.59
June 17	7106	Acid	2017	25.05	22.88	3.91	17.11
June 18	5385	Acid	1938	25.05	22.69	4.02	17.75
June 19	5537	Acid	2158	25.05	21.83	4.03	18.43
June 20	4347	Acid		25.05	20.63	4.98	24.13
June 21	5241	Acid	1729	25.05	18.27	4.25	23.28
					20.75		20.91

TABLE III.

Animal No. 2. Calf, 218 lbs.

DATE	VOL. OF URINE	REACTION TO LITMUS	TOTAL ACIDITY cc. $\frac{N}{10}$ NaOH	INTAKE N	URINARY N	URINARY AMMONIA N	AMMONIA N AS PER CENT OF TOTAL URINARY N	UREA N
20 lbs. whole milk daily.								
	cc.			grams	grams	grams		
July 7	5709	Neutral	1141	29.96	9.43	1.61	17.11	6.22*
July 8	5105	Acid	1225	29.96	11.09	1.48	13.37	7.52
July 9	4172	Neutral	1167	29.96	9.63	1.20	12.55	7.25
July 10	6017	Neutral	1443	29.96	11.87	1.41	11.88	8.75
July 11	6109	Neutral	1172	29.96	11.03	1.53	13.90	9.15
July 12	4615	Acid	1015	29.96	10.01	1.14	11.42	7.78
July 13	5302	Acid	1007	29.96	12.47	1.29	10.41	9.83
July 14	6060	Neutral	787	29.96	11.70	1.31	11.27	9.47
July 15	5741	Neutral	815	29.96	12.37	1.64	13.31	9.52
					12.40		12.80	

* Average per cent of urinary N as urea N, 76.03.

TABLE III—Continued.

DATE	VOL. OF URINE	REACTION TO LITMUS	TOTAL ACIDITY N CC. TO NaOH	INTAKE N	URINARY N	URINARY AMMONIA N	AMMONIA N AS PER CENT OF TOTAL URINARY N	UREA N
Whole milk + 11 cc. N HCl per lb. milk.								
July 16	5257	Acid	1156	29.96	12.73	2.79	21.93	8.87†
July 17	5111	Acid	1226	29.96	12.59	2.07	16.44	9.33
July 18	5642	Acid	1353	29.96	12.79	2.52	19.74	9.56
July 19	3809	Acid	1164	29.96	11.12	1.98	17.83	8.50
July 20	5833	Acid	1224	29.96	13.88	2.59	18.67	10.48
July 21	4360	Acid	697	29.96	11.29	2.43	21.57	8.40
					12.40		19.3[illegible]	
Whole milk + 16.5 cc. N HCl per lb. milk.								
July 22	5343	Acid	1068	29.96	13.31	2.54	19.09	8.62‡
July 23	5413	Acid	952	29.96	12.50	3.02	24.19	7.96
July 24	4990	Neutral	895	29.96	11.66	3.28	28.13	6.59
July 25	5165	Acid	1136	29.96	11.56	3.14	27.16	7.06
July 26	5061	Neutral	1062	29.96	12.25	4.47	36.51	6.17
July 27	5145	Neutral	778	29.96	11.16	4.45	39.91	5.15
July 28	3160	Alkaline	126	29.96	8.80	4.17	47.30	3.57
					11.49		31.75	
Whole milk + 25 cc. N HCl per lb. milk.								
August 3	4360	Acid	1090	29.96	9.64	3.84	39.88	3.98§
August 4	5890	Acid	1472	29.96	16.73	5.22	31.22	7.35
August 5	5075	Acid	1268	29.96	12.36	4.94	40.00	5.37
					12.91		37.03	

† Average per cent of urinary N as urea N, 74.11.
‡ Average per cent of urinary N as urea N, 55.83.
§ Average per cent of urinary N as urea N, 43.12.

TABLE IV.

Animal No. 3. Calf, 105 lbs. Daily averages.

DATE	VOLUME URINE	TITRATION URINE $\frac{N}{10}$	INTAKE N	FECAL N	URINARY N	URINARY NH_3 N	UREA N	PER CENT NH_3 N	PER CENT UREA N	SUM OF PER CENT	N EXCRETED	N RETAINED	PER CENT OF N RETAINED	FECAL NH_3 N
12 lbs. whole milk.														
Nov.	*cc.*	*cc.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*				*gms.*	*gms.*		*gms.*
5–10	3460	251	30.00	2.28	7.96	0.24	6.11	3.0	76.6	79.6	10.24	19.84	65.8	
12 lbs. whole milk.														
11–16	3796	321	30.00	2.54	9.09	0.39	7.09	4.2	76.5	80.7	11.63	18.32	60.8	
14 lbs. whole milk.														
17–22	4197	320	35.11	2.35	11.18	0.50	8.50	4.5	76.1	80.6	13.53	21.54	61.3	
14 lbs. whole milk, 16 cc. N HCl per lb. milk.														
23–28	4454	663	35.11	1.97	14.53	2.46	9.56	17.1	66.1	83.2	16.50	18.61	53.0	
14 lbs. whole milk, 16 cc. N HCl per lb. milk.														
Nov.–Dec. 29–4	4581		35.11	1.46	12.60	3.34	7.21	26.6	57.1	83.7	13.65	21.04	59.9	
16 lbs. whole milk, 16 cc. N HCl per lb. milk.														
5–12	5397		40.13	1.59	14.20	3.50	8.31	24.7	58.6	83.3	15.79	24.32	60.5	0.15
16 lbs. whole milk.														
13–18	4916		40.13	1.36	12.05	1.27	8.36	10.3	70.1	80.4	13.41	26.72	66.5	0.10
16 lbs. whole milk.														
19–24	5124		40.13	0.73	13.34	1.10	9.81	8.2	73.6	81.8	14.05	26.08	64.9	

TABLE V.

Record of reaction of urine, ammonia production, etc.
Animal No. 4. Calf, 125 lbs.

DATE	URINE	TITRATION URINE IN cc. $\frac{N}{10}$ HCl	N INTAKE	N URINE	NH_3 URINE N	PER CENT OF N AS NH_3
12 lbs. whole milk daily.						
	cc.		*grams*	*grams*	*grams*	
August 8.....	2693	38	21.03	6.91	0.378	5.4
August 11.....	4252	85	21.03	10.03	0.425	4.2
August 12.....	4393	339	21.03	11.89	0.475	3.9
August 13.....	3260	297	21.03	7.59	0.364	4.7
						4.5
Whole milk + 16 cc. N HCl per lb. milk.						
August 14.....	3486	873	21.03	11.38	1.084	9.5
August 15.....	3741	814	21.03	10.25	1.425	13.0
August 16.....	3288	657	21.03	8.85	1.546	17.3
August 17.....	4138	754	21.03	10.93	1.765	16.1
August 18.....	3260	575	21.03	10.99	1.716	15.6
						14.3
Whole milk.						
August 19.....	3175	635	21.03	8.43	1.676	19.8
August 20.....	3515	563	21.03	9.34	1.438	15.3
August 21.....	3288	580	21.03	10.26	1.048	10.2
August 22.....	2777	394	21.03	8.61	0.633	7.3
August 23.....	3713	232	21.03	10.68	0.666	6.2
August 24.....	2777	277	21.03	9.28	0.644	6.9
						10.9

in ammonia production in the urine as the ration became acid with mineral acidity. Accompanying the rise in ammonia production there was a compensative fall in the output of urea.

In addition, the increase of acidity in the ration did not, with but one exception (Calf 1), increase the total nitrogen excretion in the urine, as has been observed by Underhill[11] with a dog re-

[11] This *Journal*, xv, p. 337, 1913.

ceiving ammonium chloride, and by McCollum and Hoagland[12] with pigs on a starch diet plus mineral acids. It might be presumed that ammonium salts directly ingested, as in Underhill's experiments, or ammonia coupled with mineral acids after ingestion, as in McCollum and Hoagland's observations, would here likewise, under conditions of exogenous protein metabolism, stimulate nitrogen metabolism with an increased nitrogen excretion as the result. Apparently this is not generally the case on the plane of protein intake used here. Calf 1 did double its urinary nitrogen output, but a study of the data shows that it received from 1.4 to 1.7 times as much milk per pound of body weight, and as much acid to correspond, as the three other calves which do not show a like result.

It might be assumed as an explanation of these differences that in endogenous nitrogen metabolism the organism catabolizes extra tissue protein for purposes of maintaining neutrality when mineral acids are ingested, but under similar conditions, and when there is an abundant protein supply in the diet, the tissue proteins are not called upon to furnish nitrogen for such purposes. Further, the seat of ammonia production in the two cases may account for the differences observed. In endogenous nitrogen metabolism the ammonia must be produced in the tissues themselves where nitrogen catabolism would be most effectively stimulated, while in exogenous metabolism the ammonia may be largely formed in the intestine or liver and immediately excreted in combination with the acid.

This hypothesis, however, would not explain the increased nitrogen excretion on ingestion of ammonium salts, as observed by Underhill.

The very complete data gathered on Animal 3 need special comment. It might be inferred from the results of the first week on the milk-acid ration that an acid ration would decrease nitrogen storage. This was apparently true as an average result for the first week after the change from the neutral to the acid diet, but such a condition did not remain permanent. In the two following weeks on an acid ration the nitrogen storage was again approximately that of the basic diet.

[12] *Ibid.*, xvi, p. 299, 1913-14.

While there was a rise of urinary ammonia with all animals as increasing quantities of mineral acids were ingested, there was also a rise of acidity to phenolphthalein and a more general acidity to litmus, although urines neutral to litmus were even here occasionally produced. The data presented confirm the work of Winterberg; namely, that there is no distinction between omnivora and herbivora in their ability to maintain tissue neutrality by the use or production of ammonia under conditions of acidosis. These data apply specifically, however, to conditions of exogenous nitrogen metabolism.

Although not specifically germane to the subject being discussed, attention should be called to the large storage of milk protein nitrogen observed with this species when fed this class of proteins. A retention of 60 to 65 per cent of the absorbed nitrogen was observed, while with somewhat older animals[13] of the same species a retention of 20 to 25 per cent of the absorbed alfalfa hay or corn grain nitrogen was all that could be obtained. In the latter case, however, the proteins constituted about 11 per cent of the ration, while in the former case they approximated 25 per cent. Very probably, however, increasing the concentration of alfalfa or corn nitrogen in the ration would not have materially improved the percentage of retention.[14]

Endogenous nitrogen metabolism.

The fact that an herbivorous animal can control conditions of acidosis on rations with a considerable protein content is well established by the above data, but it was of further importance to determine whether this class of animals could exercise such control on a level of protein intake reduced to a possible minimum. Under ordinary conditions of protein metabolism ammonia may be derived for purposes of maintaining neutrality from the regular processes of digestion, from the putrefactive processes in the intestine, or from the normal metabolism of the tissue cells. It is very probable that on ordinary acid rations the first two sources are ample enough to provide ammonia for purposes of maintaining neutrality.

[13] Hart, Humphrey and Morrison: this *Journal*, xiii, p. 134, 1912.
[14] McCollum: *ibid.*, xix, p. 323, 1914.

To determine whether this class of animals (calves), which are almost always on alkaline rations, had lost the power of controlling acid ingestion by the production of ammonia in the tissues, it was necessary to reduce the plane of protein intake to as low a level as possible. The anatomical structure of this species makes it imperative that a roughage accompany the ration very early in life. This made it extremely difficult to reduce the protein metabolism to an endogenous level. At first, attempts were made to feed starch alone as a water suspension to satisfy the energy requirements of the animal. Consumption was sufficient but it passed through the animal too quickly and was undigested. No diarrhea, however, resulted. When a soluble carbohydrate, as milk sugar, was mixed with the starch for the purpose of compensating for the undigested starch, diarrhea resulted and consequently this procedure could not be followed. Failure of consumption followed an attempt to serve the starch as a paste, and even when this paste was mixed with filter paper, for purposes of aiding retention in the tract, inadequate consumption resulted. An attempt was made to use dry starch and filter paper, and while the paper aided in checking a too rapid passage of the starch through the tract it was impossible to get the animal to eat a sufficient quantity to cover its energy requirements. Finally success followed the use of cut wheat straw and dry starch. While this introduced a small amount of nitrogen, yet as can be seen in Table VI which follows, the fecal nitrogen was always greater than the intake and decreased as the bulk of the straw was decreased. In fact, had we been successful in the use of filter paper in the diet, the fecal nitrogen would still have been considerable, due to the copious secretions poured into the tract by this class of animals. A preliminary period of four days preceded the actual collection of the records; acid was given in increasing quantities by means of a bottle, and from ten to twelve pounds of water were allowed daily after the fifteenth of June. Before this time it was given in somewhat greater quantities.

Following the records secured on an approximate endogenous nitrogen level, a period of high protein intake (milk) follows. Besides complete nitrogen balances and urea determinations there are given some data on calcium and phosphorus balances.

From the data presented it appears that this class of animals is likewise capable of producing ammonia in the tissues for purposes of maintaining neutrality when conditions arise requiring it. While the rise in urinary ammonia nitrogen excretion did not progressively increase with increasing acid ingestion, nevertheless there was a marked rise in the first acid period and another when the intake reached 400 cc. of N HCl. This failure of a continuous rise may be correlated with the increased output of urine, accompanying a large water consumption and a tolerance for a more acid urine. When the consumption of water was decreased and the ingestion of acid increased there was another marked rise in urinary ammonia nitrogen, reaching 34.8 per cent of the total urinary nitrogen.

The source of the urinary ammonia with the high acid ingestion may still be problematic. The presumptive evidence is that it originated in the tissues. While there was always a considerable amount of fecal nitrogen, due to the liberal intestinal secretions accompanying the ingestion of a roughage, yet the ammonia content of the feces was very constant. When the demand for ammonia was greatest, namely, in the high acid period, the ammonia content of the feces remained practically at the same level as during a no acid period. Had the ammonia had its origin in the intestine, the fecal ammonia should have been materially reduced during the high acid period. As this did not occur it makes it very probable that the urinary ammonia originated in the tissues during the high acid period.

Accompanying the high acid ingestion and increased ammonia production there was a corresponding decrease in urea production. This was practically a quantitative result, as seen in the constancy of the sums of the percentages of urea nitrogen and ammonia nitrogen.

With this animal there was no stimulation to protein metabolism with the rise in production of ammonium salts, as has been observed by Underhill on administering ammonium salts to a dog, and by McCollum and Hoagland on giving a pig mineral acids in endogenous protein metabolism. Whether this is a constant and fundamental difference in the behavior of the several species or whether some other factor was operative in this experiment is difficult to say. Possibly the greater dilution of acid incident to

TABLE VI.

Records of nitrogen, calcium and phosphorus

DATE	N HCl ADDED TO RATION	RATION	INTAKE N	FECAL N	URINE N	FECAL NH_3 N	URINE NH_3 N	UREA N	PER CENT N AS NH_3	PER CENT N AS UREA
May	cc.		gms.	gms.	gms.	gms.	gms.	gms.		
31	0	3 lbs. straw; 2 lbs. starch; salt	5.54	11.44	8.39	0.51	1.49	4.36	17.07	51.9
June										
1	0		5.54	12.70	6.31	0.48	0.42	3.67	6.6	58.1
2	0		5.54	9.76	6.63	0.32	0.66	3.66	9.9	52.2
3	0		5.54	21.02	3.94	0.70	0.56	2.09	14.2	53.0
4	0		3.70	13.02	8.27	0.48	1.23	4.36	14.8	52.7
Average			5.17	13.59	6.71	0.50	0.87	3.63	12.6	53.6
5	50	2 lbs. straw; 2 lbs. starch; salt	3.70	7.48	7.42	0.23	1.38	3.86	18.5	52.0
6	100		3.70	13.31	7.25	0.43	1.45	3.85	20.0	53.1
7	100		3.70	10.73	8.85	1.03	2.72	3.64	30.5	41.1
8	100		3.70	10.44	6.34	0.44	1.60	2.56	30.7	40.4
9	100		3.70	11.90	6.67	0.35	1.33	3.15	19.9	47.2
10	100		3.70	8.33	6.19	0.20	1.00	3.06	16.1	49.4
Average			3.70	10.94	7.06	0.49	1.62	3.25	23.4	46.2
11	200		3.70	11.56	5.88	0.27	1.03	2.92	17.5	49.8
12	200		3.70	8.60	6.49	0.22	1.08	3.45	16.6	53.2
13	200		1.85	6.64	6.69	0.09	1.11	3.49	16.6	52.2
14	200		1.85	9.29	5.89	0.47	1.18	2.88	19.9	48.8
15	200		1.85	7.01	7.01	0.31	1.30	3.56	18.4	50.8
16	200		1.85	7.07	6.83	0.17	1.37	3.53	20.0	51.7
17	200		1.85	6.17	6.52	0.19	1.19	3.46	18.2	53.1
Average			2.38	8.05	6.48	0.25	1.18	3.33	18.2	51.4
18	300	1 lb. straw; 1 lb. starch; salt	1.85	6.90	5.98	0.17	1.54	2.65	25.7	44.3
19	300		1.85	11.92	8.09	0.15	1.15	4.24	18.6	52.4
20	300		1.85	5.55	5.57	0.20	1.45	2.57	26.0	46.1
Average			1.85	8.12	6.54	0.17	1.50	3.15	23.4	47.6
21	400		1.85	5.19	6.94	0.35	2.39	2.70	34.4	39.0
22	400		1.85	4.32	7.43	0.14	2.51	3.03	33.7	40.7
23	400		1.85	5.35	6.90	0.46	2.51	2.35	36.3	34.1
Average			1.85	4.95	7.09	0.32	2.47	2.69	34.8	37.0
24	200	6 lbs. milk; ½ lb. straw; ½ lb. starch	12.62	4.82	9.66	0.27	1.51	5.68	15.7	58.8
25	0	15 lbs. milk; 1 lb. straw; 1 lb. starch	31.10		24.13		5.73	11.94	23.6	49.4
26	0		31.10		20.22		3.48	14.13	17.2	69.8
27	0		31.10	6.61	22.11	0.40	3.61	15.69	16.3	70.9
28	0		31.10	7.64	19.49	0.45	3.20	13.76	16.4	70.6
29	0		31.10	3.32	17.01		2.88	12.10	16.9	71.1
Average			31.10	5.86	19.71	0.42	3.29	13.92	16.7	70.6

balances with calf. Low nitrogen intake.

Urea + NH_3 N per cent of total N	Balance N	Urine vol.	Titration N cc. T6	CaO feed	CaO feces	CaO urine	CaO balance	P_2O_5 feed	P_2O_5 feces	P_2O_5 urine	P_2O_5 balance
	gms.	cc.		gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.
69.6	−14.29	4270				0.307				0.461	
64.7	−13.47	2104				0.193				0.264	
62.1	−10.85	2920				0.257				0.233	
67.2	−19.42	2387				0.176				0.218	
67.5	−17.59	3676									
66.2	−15.13	3071				0.233					
70.5	−11.73	3198									
73.1	−16.86	1964									
71.6	−15.88	5389									
71.1	−13.08	6400									
67.1	−14.87	8084									
65.5	−10.82	6876									
69.7	−14.30	5762									
67.3	−13.74	6876									
70.8	−11.39	6539									
69.8	−11.48	7950									
68.7	−13.33	5237									
69.2	−12.17	4714	394	1.344		1.074		4.845		1.933	
71.7	−12.05	5163	412	1.344	0.296	0.909	+0.139	4.845	0.310	1.601	+2.934
71.3	−10.84	3231	490	1.344	0.268	0.749	+0.327	4.845	0.388	1.931	+2.526
69.8	−12.15	5673	432	1.344	0.282	0.911	+0.151	4.845	0.349	1.822	+2.674
70.0	−11.03	3339	480	1.344	0.277	0.694	+0.373	4.845	0.377	1.940	+2.528
71.0	−18.16	5584	802	1.344	0.419	1.206	−0.281	4.845	0.749	3.116	+0.980
72.1	−9.27	3260	494	1.344	0.270	0.839	+0.235	4.845	0.384	2.073	+2.388
71.0	−12.81	4061	592	1.344	0.322	0.913	+0.109	4.845	0.503	2.376	+1.966
73.0	−10.28	4393	577	1.344	0.287	1.353	−0.296	4.845	0.383	1.985	+2.477
74.4	−9.91	4734	570	1.344	0.255	1.831	−0.742	4.845	0.339	1.846	+2.659
70.4	−10.40	2097	571	1.344	0.275	1.655	−0.586	4.845	0.321	1.160	+2.364
72.9	−10.19	3741	572	1.344	0.272	1.613	−0.541	4.845	0.347	1.664	+2.834
74.5	−1.86	1445	597	4.712	0.216			8.267	0.223	1.941	
73.0		3543		11.463				19.520			
87.0		5811	1229	11 463				19.520			
87.2	+15.60	4195	2066	11 463		trace		19.520		19.009	
86.6	+3.97	2976	1727	11.463		trace		19.520		10.454	
88.0	+10.77	3032	1428	11.463		trace		19.520		11.174	
87.2		4003	1612	11.463				19.520		13.546	

a large water consumption rendered the concentration of ammonium salts in this experiment insufficient to induce the stimulation observed in the experiments mentioned.

Some rather remarkable results were observed when this animal was changed from the high acid-low nitrogen ration to a milk diet. Contrary to what might be expected there was a marked increase in urinary acidity; accompanying this there was almost complete absence of calcium in the urine, but a large output of phosphorus by that channel. The urinary calcium and phosphorus were rather low during the straw-starch period but were considerably increased during the straw-starch-acid periods. The fact that the urinary calcium was not increased by raising the acid ingestion from 200 cc. to 300 cc. of N HCl supports the idea that this urinary calcium and phosphorus in these periods were *mainly*, if not wholly, coming from the ingested straw. Had it had its origin in the bones, one would have expected a progressive rise in urinary calcium with increased acid intake. When, however, 400 cc. of acid were given the animal, then a marked increase in urinary calcium excretion again resulted. Since there was no rise in urinary phosphorus in this period it may be inferred that the extra calcium was now coming from the calcium carbonate-calcium phosphate complex of the bones. In other words, not until there was a high intake of acid were the bones drawn upon for bases, and then only the calcium carbonate portion.

The marked rise in urinary acidity accompanying the milk diet with the disappearance of calcium from the urine is possibly due to the rapid storage of calcium on cessation of a high acid diet. This rapid storage of bases and also probably rapid storage of nitrogen by an animal with a strong growth impulse left a considerable excess of phosphorus as a residue from the food ingested for excretion in the urine. This large excretion of phosphorus as an acid salt would account for the observed rise in urinary acidity.

DISCUSSION.

What effect the constant presence of ammonium salts in considerable quantities may have in the organism is not to be answered except by direct observations of the influence of acid rations on the life of the animal.

While in endogenous nitrogen metabolism (dog and pig), ammonium salts may cause a stimulated protein catabolism, yet on a protein-rich diet (calves) no such effect was observed. Neither on an approximate endogenous level, accompanied by a high intake of mineral acid, was an increased nitrogen excretion to be observed with the calf. It can hardly be claimed then that there would be interference with protein storage if a ration carried a fair amount of protein, but was of acid character, especially with the degree of acid inherent in natural feeding materials.

In support of the view that a ration, if otherwise satisfactory, may be acid in character without apparent detrimental effect to the animal, the following observations, partly from unpublished records of this laboratory, have been made. Two cows receiving their nutrients from the corn plant, and with a protein intake of approximately 12 per cent, have received daily for two years a mixture of sulphuric and phosphoric acid. The daily quantity of acid used was 30 cc. of 1.88 phosphoric acid and 16 cc. of 1.84 sulphuric acid per 14 pounds of feed. These are quantities sufficient to throw the relation of bases to acids in the ration on the acid side. That this was accomplished was shown by the acid reaction of the urine, otherwise neutral or alkaline, and by the high urinary ammonium content. The ammonium nitrogen in the urine varied from 7 to 14 per cent of the total nitrogen in one individual and 19 to 23 per cent in the other on the days examined. On the corn ration without acid the urinary ammonium nitrogen was never above 2.5 per cent, yet these animals remained in splendid condition and one of them produced a normal, strong calf of 73 pounds in 1914.

Swine fed mixed or a single grain ration plus tap water have been grown from a weight of 40 to 50 pounds to 350 pounds. These rations were of acid character, the urinary nitrogen as ammonia rising as high as 12 per cent of the total urinary nitrogen. It must be stated that while growth could be secured, yet reproduction was not normal. In some cases, however, when the animal was placed on the ration after reaching 200 pounds' weight a normal reproduction could be secured. Whether the difficulties with swine should be attributed at all to the acid character of the ration, or to other as yet undetermined factors when grains

alone make up the ration, is not wholly clear. In this laboratory[15] rats have been grown on egg yolk (an acid diet) with apparently normal growth and vigor and reproduced normal young. They have also been kept on an artificial ration, with a mineral content like that of egg yolk, which would give the diet an acid character, and under such conditions they have produced considerable growth.

It is very probable, then, that where the ration or diet is otherwise satisfactory the degree of acidity inherent in natural foods (here reference is only made to the balance of acid and base elements) is less harmful than is generally supposed. It would, however, be highly desirable if more data on the complete life cycle of animals could be presented in order that this matter might be incontrovertibly established. There is always a possibility of the weak individual and until it has been shown that the less vigorous will tolerate acid rations with perfect impunity we are not warranted in making too rigid and sweeping conclusions.

SUMMARY.

Acid rations fed to swine (omnivora) or calves (herbivora) occasion a rise in urinary ammonia with a compensative fall in output of urea. Presumably on a normal level of protein intake a part of the ammonia, produced either in the intestine or liver, combines with acids and is excreted as the salts of these acids. This power to help maintain neutrality by the production or use of ammonia is apparently very general in all mammals.

Ammonia production, under conditions of exogenous protein metabolism, does not occasion an increased nitrogen excretion or an interference with protein storage.

In herbivora (calves) approximate endogenous nitrogen metabolism, accompanied by mineral acid ingestion, likewise occasions a rise in urinary ammonia, but does not, on the level of acid used, cause a rise in protein catabolism, as has been observed with dogs and swine. This may be due in this experiment to a greater dilution of the ammonium salts incident to a large consumption of water by this class of animals.

[15] McCollum and Davis: *Proc. Amer. Soc. Biol. Chem.*, this *Journal*, xiv, p. 128, 1913.

Data are also given on calcium and phosphorus metabolism during both neutral and acid periods of low nitrogen intake, as well as on a period of high nitrogen intake. Very probably the skeleton was not drawn upon for calcium during the period of lowest acid ingestion. Only on a high acid ingestion did it appear probable that decalcification of the bones began and then only a withdrawal of calcium carbonate.

From the records submitted on growth and reproduction, it is believed that natural acid rations, if otherwise satisfactory, are as effective for growth or reproduction as those of basic character. However, until it has been shown conclusively that less vigorous individuals will tolerate acid rations with perfect impunity, we are not warranted in making too sweeping conclusions.

THE EFFECTIVE PRINCIPLE IN THYROID ACCELERATING INVOLUTION IN FROG LARVAE.

By MAX MORSE.

(From the Department of Physiology, University of Wisconsin.)

(Received for publication, September 24, 1914.)

The interesting and significant observations of Gudernatsch[1] on the accelerating action on metamorphosis of thyroid fed to frog larvae are of peculiarly potent suggestion in the studies which the writer has been making[2] of involution; for we have in this agent something which reduces the time of involution more than three-fifths, exerting, therefore, a power over the speed of tissue absorption, along with other physiological phenomena, unknown in any other case. It is desirable to determine, if possible, to what this effect is more specifically due. The question is: Can any one factor or group of factors in thyroid be determined which are responsible for the effect of the intact gland substance?

The chemical components of thyroid have been widely studied, and it is now possible to give a fairly complete invoice of these compounds, as follows:

Since the time of Baumann (1895) the components have been divided into those which bear iodine and those which are free from this halogen; for in that year the discovery was made that iodine is a constant and normal constituent of mammalian thyroid.[3] Later Oswald,[4] following the studies of Bubnow[5] upon thyroid proteins, determined that the iodine was associated in some way with the "thyreoprotein" of Bubnow, which Oswald showed to be a globulin, and termed "thyreoglobulin." In the meantime, Baumann had shown that after hydrolysis of thyroid with acid, a melanic mass, not a protein, resulted, which bore iodine; and this he termed "iodothy-

[1] *Centralbl. f. Physiol.*, xxvi, p. 323, 1912; *Arch. f. Entwicklungsm.*, xxxv, p. 457, 1912; *Amer. Journ. of Anat.*, xv, p. 431, 1914.

[2] *Proc. Soc. Exp. Biol. and Med.*, x, p. 31, 1912–13; xii, p. 45, 1914; *Biochem. Bull.*, ii, p. 546.

[3] Baumann: *Zeitschr. f. physiol. Chem.*, xxi, p. 319, 1895–96.

[4] *Ibid.*, xxvii, p. 15, 1899.

[5] *Ibid.*, viii, p. 1, 1883.

rin." From the work of Nürnberg[6] and others it appears that this complex mass is a group of protein hydrolysis products such as amino-acids, and that the iodine is associated with the "*Baustoine*" in some way as yet undetermined. It is probable that at least di-iodotyrosine and iodotryptophane exist in this way.

Aside from the iodine-bearing components, many others have been discovered, such as nucleoalbumin,[7] thyreoantitoxin,[8] lecithin, etc. For the present purpose the iodine-bearing compounds, together with nucleoalbumin and lecithin alone, will be considered; for the preliminary experiments upon which the following ones are based showed that the problem is approachable without considering the rôles of any other constituents.

The function of the thyroid has been variously interpreted, but it is possible to arrange the different theories for the present purpose in two categories; namely, the detoxicating theory of Notkin[9] and Blum,[10] which is now untenable; and, secondly, the secretion theory of Baumann,[11] Roos,[12] Oswald[13] and other investigators, which is applicable to most of the latter day work. Distinctly, the present set of observations are not interpretable along the lines of the "*Entgiftungstheorie*," and the work of Ewald,[14] and many others make it improbable that the thyroid functions essentially as a detoxicating organ, although some such function is apparent by the work of Hunt[15] and others.

The secretion theory may in turn be divided into two parts according as the iodine-content is emphasized or as some other component is considered. Baumann's school (Oswald, Roos, *et al.*) holds that the halogen is responsible for the physiological effects of thyroid, while von Fürth[16] has shown that some of the effects, *e.g.*, vasomotor reactions, can be produced by choline, although this amine derivative is found widely distributed throughout the animal body, so that there is nothing specific in thyroid choline.

Aside from the vasomotor reactions (lowering of blood pressure, etc. effect upon pulse-frequency and upon muscular tone), thyroid preparations exert an effect upon the nitrogen balance. All of these effects can be enhanced negatively by extirpation, and they also appear in such diseases as cretinism, myxoedema, etc., where concomitant pathological changes

---

[6] *Beitr. z. chem. Physiol. u. Path.*, x, p. 125, 1907.
[7] Gourlay: *Journ. of Physiol.*, xvi, p. 23, 1894.
[8] Fränkel: *Wiener med. Bl.*, 1896, No. 13.
[9] *Wien. med. Wochenschr.*, 1895, p. 824.
[10] *Berl. klin. Wochenschr.*, 1898, p. 950, xxxv.
[11] Baumann and Goldmann: *Münch. med. Wochenschr.*, xliii, p. 1153, 1896.
[12] Roos: Habilitationsschrift, Freiburg i. B., 1897.
[13] Oswald: Habilitationsschrift, Strassburg, 1900.
[14] *Z. klin. Wochenschr*, 1887, No. 2.
[15] This *Journal*, i, p. 33, 1905.
[16] von Fürth and Schwarz: *Arch. f. d. ges. Physiol.*, cxxiv, p. 113, 1908.

of the thyroid occur. Implantations of living thyroid, subcutaneous injections of thyroid substance, feeding *per os* of thyroid preparations and also of certain iodized proteins, such as iodized Witte peptone, iodized casein, etc., all serve to allay the symptoms of these diseases.

With this brief review of the thyroid, we may proceed to the experiments which the writer has performed, and later the question will be raised as to whether any of the functions of mammalian thyroid cause the effects which Gudernatsch determined on feeding thyroid to frog tadpoles.

The larvae of the common species *Rana pipiens* were used throughout. A single batch of several hundred was brought into the laboratory and controls were conducted to each experiment in two series. In one the larvae were fed upon fresh water algae (*Spirogyra*, etc.); in these controls metamorphosis occurred within about two weeks. The second control consisted of larvae fed with Parke Davis and Company's Desiccated Thyroid Extract and Armour's Thyroid Tablets.[17] Metamorphosis occurred typically in three days when the room temperature stood at 30°C., but if it fell to 20°C., as in some instances, the development was delayed for a few hours longer, but never later than the fourth day. The difference between the maturing time of these two controls gave definite data with regard to the efficacy of the various reagents used. Artesian spring water was used for the cultures throughout. The experiments were performed in 25 cm. Petri dishes, and a liter of water was used in each case.

Besides the desiccated thyroid, some of the experiments were performed by the use of fresh thyroid gland substance from Cudahy Company.

Is the choline content responsible for the effects of intact thyroid? Aside from the minute amount of choline in the included blood of the thyroid gland tissue, this substance occurs only in lipoids, that is "lecithin." The lipoids were removed by cold and hot ether and alcohol, and the residue after extraction was fed to the larvae; the larvae metamorphosed within three days, showing that the removal of the lecithin and neutral fats exerted no appre-

[17] Prof. E. V. McCollum has called my attention to the fact that recently another worker has reported the successful use of desiccated thyroid and tablets (West: *Science*, N. S., xxxix, p. 918, 1914).

ciable effect on the time of involution. The residue obtained after evaporating the ether was fed and no effect was produced. Hen's egg lecithin, obtained after the method of Roaf and Edie,[18] gave negative results when fed to the larvae. We may conclude that choline exerts no appreciable effect upon the larvae as far as time of metamorphosis is concerned. It is doubtful whether blood system changes figure very markedly in metamorphosis; for in 1912 I ligated the larger vessels going to the tail of an individual belonging to the species *Rana catesbiana* and no appreciable effect was noted; neither was metamorphosis accelerated by ligations in normal larvae nor was the process inhibited in ligated individuals which had started to metamorphose.

Do inorganic iodine preparations produce the effects of intact thyroid? "Metallic" iodine (Merck's "resublimed scales"), which is soluble in water at 30°C., in about 0.03 per cent, was placed in the water in which the larvae rested. After a week controls were normal, the thyroid-fed ones were metamorphosed, and the larvae of the present experiment remained undeveloped. Potassium iodide, iodine in potassium iodide, methyl iodide, iodobenzoic acid, iodoxy-benzoic acid were all used with similar negative effects. Therefore, as far as these experiments are concerned, inorganic iodine preparations are ineffective in producing the effects of intact thyroid.

Do iodine-bearing sea algae produce the effect? Through the kindness of Professors H. C. Bradley and J. E. Rush, I obtained several species of marine algae from Woods Hole, Massachusetts. The following species were used in the experiments: *Fucus vesiculosus*, *Chondrus crispus* and *Laminaria sp.* The fronds were soaked in distilled water and dialyzed against water to remove the adhering salts. The material was fed to the larvae and they were observed to eat it. The results were negative throughout, signifying that whatever the mode of retention of the iodine in the plants, it is not efficient in producing the effects of thyroid. Dorwald,[19] Chatin[20] and others have shown that the iodine in these plants is bound with alkali, such as potas-

[18] *Thompson Yates and Johnston Laboratory Reports*, vi, p. 1.

[19] *Compt. rend. de l'Acad. des Sci.*, xxviii, p. 66; *Journ. of Pharm.*, (3), xv, p. 209.

[20] *Journ. Pharm.*, (3), xvii, p. 418.

sium. Inasmuch as there is no question that the iodine is made available during the process of digestion by the larvae, for their digestive enzymes are capable of handling cellulose in quantities, the suggestion offered by our knowledge of the way in which the iodine is linked in plants is interesting, pointing as it does to the general fact brought out above, that inorganic iodine preparations are inefficient.

Does starch iodate produce the result of thyroid? Starch iodate was prepared, washed with water and with ethyl alcohol, dried on a Buchner funnel and fed to the larvae. The results were negative. Duclaux[21] believes that iodine is held in starch solution in colloidal form, by adsorption, while Meineke[22] has found that hydrogen iodide is a necessary adjunct, although this has been disputed by Rouvier.[23] At any rate, whether the iodine is in colloidal form, or whether it is in chemical combination, in the presence of HI, the fact remains that it is not available for functioning like thyroid.

Do iodine compounds, other than those derived from thyroid, produce the effects? Kurajeff's[24] blood-protein iodine compound, sold on the market by Parke Davis and Company as "iodalbim,"[25] was used in capsule form. The brown powder was suspended in the culture water and taken in passively by the larvae. The results were positive, running in time quite along with the thyroid controls, with but a few hours lag.[26] Besides iodized blood-protein, iodized hen's egg lecithin was used, the results being negative. Merck's dried egg-albumin was iodized at 40°C. in a thermostat and fed to the larvae after washing and treating with Na_2CO_3 to remove the hydrogen iodide which forms. The results were negative, although unilateral paralysis occurred among the tadpoles.

Aside, then, from iodized blood-albumin, the results of feeding iodized compounds were negative. However, the fact that the

[21] *Compt. rend. de l'Acad. des Sci.*, lxxiv, p. 533.

[22] *Chem. Zeitung*, xviii, p. 157, 1894.

[23] *Compt. rend. de l'Acad. des Sci.*, cxiv, p. 128, 1892.

[24] *Zeitschr. f. physiol. Chem.*, xxvi, p. 462, 1898-99.

[25] That this is essentially after the formula of Kurajeff appears from the data of the department of investigation of the American Medical Association (see *New and Non-Official Remedies for 1913*, p. 136).

[26] At 30°C. the lag was about eight hours. The thyroid larvae had metamorphosed during the third day.

iodized blood-albumin gave positive results, comparable with thyroid itself, is of striking interest. Taken in conjunction with data to follow, it points to a protein linkage of some sort. In regard to halogenated fats, the experience of Coronedi[27] with iodized lipoids showed that while these compounds acted favorably in cases of cretinism, myxoedema, etc., the effects were negative after thyreoidectomy.

What fractions of thyroid substance, if any, produce positive results? Thyreoglobulin was prepared after the method of Oswald:[28] The desiccated powder was treated with half saturated solution of ammonium sulphate over night under toluol, in an ice-chest at about 10°C. In the morning the preparation was filtered, the residue being again treated with half saturated $[NH_4]_2 SO_4$, and again filtered. The residue was suspended in water and precipitated with dilute acetic acid, the precipitate being caught upon a Buchner funnel and again suspended and precipitated with 95 per cent ethyl alcohol. The precipitate was washed upon the funnel, dried in air at room temperature and used for the experiments in this way. The results were positive, the time running with that of the controls with intact thyroid.

Baumann's iodothyrin[29] was then studied. The preparation was made according to the method of that investigator: Thyreoglobulin, prepared as above, was digested for three days with 10 per cent H_2SO_4 upon an electric stove under reflux condenser. The insoluble black mass was filtered off, washed free from acid, taken up by water and, after the water had been evaporated off, the residue was ground in a mortar with lactose. The mixture of lactose and thyroid substance was then taken up in hot water and filtered through a dry filter. By several treatments of hot water the lactose was entirely removed. The residue was dried over H_2SO_4 and in this form it was used by being thrown into the culture water. About a gram to the liter of water gave positive results running parallel with the intact substance.

From these two sets of experiments, it is clear that the iodine-bearing portions of the thyroid substance, that is, thyreoglobulin

[27] *Atti d. Accad. med.-fis. fiorentina*, 1903.
[28] Oswald: *loc. cit.*
[29] Baumann: *loc. cit.*

and iodothyrin, give results which cannot be distinguished from those obtained by feeding the desiccated powder or tablets.

Do iodized amino-acids produce positive results? 3-5-Di-iodotyrosine was prepared after the method of Wheeler and Jamieson,[30] as modified[31] by Oswald: With a specimen of tryptic digestion, from which the tyrosine was removed by recrystallization from hot water and alcohol, the crude tyrosine was dissolved in $\frac{N}{1}$ NaOH,[32] treated in a freezing mixture with pulverized iodine scales, stirring being maintained throughout until the mass began to gelatinize; later, on the addition of more iodine, fine crystals appeared. The above behavior pointed to the identity of the tyrosine used as *l*-tyrosine, for upon iodizing *d-l*-tyrosine, no gelatinous stage intervenes. The precipitate formed was washed on a dry filter with ice-water and some of the iodized material was recovered from the filtrate. The final united residue was dissolved in $\frac{N}{10}$ NaOH, precipitated with acetic acid and finally crystallized out with hot 70 per cent ethyl alcohol. The dried material thus obtained was fed to the larvae.

The results were positive, the time running behind that of the tadpoles fed with intact thyroid substance by two days, the latter metamorphosing on the third day. Inasmuch as the controls in pure water showed no evidence of metamorphosis after nearly a week from the time of the completion of this experiment, I feel that the conclusion is abundantly warranted that 3-5-di-iodotyrosine gives positive results. A small quantity only of the iodized amino-acid could be obtained, so that, perhaps with a larger amount, the time element would have approached more nearly to that of the control.

Strouse and Voegtlin[33] found that 3-5-di-iodotyrosine did not give favorable results with thyreoidectomized dogs, nor in the cases of goitre and myxoedema in human subjects, but nothing is more certain in reviewing the investigations which have been carried on upon thyroid constituents than that the effects vary with the subject. In the present instance we are unable to say that

[30] *Amer. Chem. Journ.*, xxxiii, p. 365, 1905.

[31] *Zeitschr. f. physiol. Chem.*, lix, p. 321, 1909.

[32] Probably some leucine remained in the preparation; but at the temperature used, 0°C., this would not become iodized.

[33] *Journ. of Pharmacol. and Exp. Ther.*, i, p. 123, 1909-10.

there is any similarity in the various phenomena which thyroid produces in frog larvae and in pathological conditions, such as *thyreoidectomy*, Basedow's disease, cretinism, tetany, etc., found in mammals. Some conditions are apparently similar, as, for instance, the greater susceptibility to unfavorable external stimuli, but farther than these one may not legitimately draw parallels.

It is difficult and indeed impossible to indicate exactly what effect the iodized compounds exert upon the larvae. One promising suggestion is revealed when we remember that Marbe[34] has shown that the opsonic index of rabbits is raised by feeding thyroid derivatives bearing iodine. There is no question but that phagocytosis plays a rôle, even if not a primary and instigating rôle, in involution of the tadpole's tail,[35] and perhaps the iodized compounds enhance the efficiency of these cells in producing atrophy. Of course this still leaves unexplained the various and wide-spread processes of differentiation which are likewise stimulated; but it may be that, as Weismann[36] has said in another reference, "Die Rückbildung überflüssig gewordener Theile (*i.e.*, the larval organs), ist also Bedingung des Fortschrittes,"—the degenerative changes may be incitants of profound changes resulting in what we call metamorphosis.

Another suggestion may be made. Looss (l.c.) has observed appearances of dissolution in the tissues of metamorphosing larvae of the frog before entrance of the phagocytes, and this is even more evident in other involuting tissues, such as occur in insects during metamorphosis. Again, as I have shown,[37] differential counts of leucocytes of phagocytic kind in normal and absorbing individuals fail to show the differences which we have reason, from mammalian studies, to postulate for absorbing tissues, where the process is one of very active phagocytosis. Histological studies reveal the fact that the so called phagocytosis is more probably due to extravasation of both reds and whites when the tissues break down than to active immigration of the phagocytes; more-

[34] *Compt. rend. soc. biol.*, lxix, p. 355; lxxii, p. 802.

[35] Cf. Metchnikoff: *Biol. Centralbl.*, iii, p. 560; Mercier: *Arch de Zool. exper. et gén.*, (4), v, p. 1; Looss: *Ueber die Degenerations-Erscheinungen im Thierreich, etc.*, Preisschrift von der fürstlichen Jablonowski'schen Gesellschaft in Leipzig, xxvii, Leipzig, B. S. Hirzel.

[36] *Ueber den Rückschritt in der Natur*, p. 5.

[37] *Proc. Soc. Exp. Biol. and Med.*, x, p. 31, 1912-13.

over, there is a striking paucity of polynuclear leucocytes in these sections. Lastly, dialysis experiments with distilled water and with serum from adult frogs upon normal and absorbing tissues show that the former are negative to ninhydrin, the latter positive, pointing to the accumulation of α-amino-acids and hence to autolysis, as do the foregoing observations. Now if it is a matter of autolysis, which is by no means certain,[38] the work of Kepinow[39] is suggestive, for he finds that on addition of iodine compounds (KI, etc.) to *in vitro* and *in vivo* autolysis, the process is accelerated markedly. The chief difficulty with this suggestion is that inorganic preparations give positive results, whereas in my work, only organic-bound iodine is available; but it might be that an iodization of the proteins took place during autolysis in Kepinow's case.[40]

The following conclusions seem to be warranted:

1. The specific effect of thyroid, discovered by Gudernatsch, in shortening the period of metamorphosis in frog larvae concerns iodine, which is associated in some way with the amino-acids composing the complex iodized globulin of the thyroid.

2. As has been determined for mammals, inorganic iodine preparations do not produce the results.

3. Not only thyroid tissue and extracts are capable of inducing Gudernatsch's results; iodized blood-albumin serves to produce the same results; *i.e.*, it is not a specific thyroid reaction.

4. The iodine of algae is not available in this instance, owing doubtless to the fact that it is not in organic association.

5. Colloidal iodine, such as exists in starch, is not available.

6. In the light of our knowledge of phagocytosis in metamorphosing frog larvae, the suggestion is made that perhaps the heightening of the opsonic index, which is known to result in other organisms after feeding thyroid, is responsible for the effects on the larvae.

[38] See a paper by the writer in the *Proc. Soc. Exp. Biol. and Med.*, xi, p. 184, 1913, in which *in vitro* experiments with absorbing and non-absorbing tissue fail to reveal any difference by methods of precipitation, cryoscopy, conductivity and amino-acid determinations by the gasometric method and by that of formol titration.

[39] *Biochem. Zeitschr.*, xxxvii, p. 238, 1911.

[40] Schryver (*Journ. of Physiol.*, xxxii, p. 159, 1905) found that liver autolysis was accelerated by feeding thyroid; Wells, on the other hand, found that thyroid extract when added to an *in vivo* autolysis culture exerted no effect.

IS THE ANTAGONISTIC ACTION OF SALTS DUE TO OPPOSITELY CHARGED IONS?

By JACQUES LOEB.

(*From The Rockefeller Institute for Medical Research, New York.*)

(Received for publication, September 29, 1914.)

I.

The writer has shown in earlier papers[1] that a pure solution of NaCl of that concentration in which this salt occurs in sea water kills the newly fertilized eggs of the marine fish *Fundulus* so rapidly that they are unable to form an embryo, and that the addition of a small amount of certain salts with a bivalent metal (*e.g.*, Mg, Ca, Sr, Ba, Pb, Zn, Mn, Co, etc.) prevents this injurious action of NaCl. That this result could not be explained on the assumption that the egg needs Mg or Ca or Zn for its development was proven by the fact that in twice distilled water the same eggs not only can develop perfectly normally, but that the young fish can hatch and live. The only inference was that the pure NaCl solution in that concentration in which it is contained in the sea water injures the egg, while the addition of $CaCl_2$ (or the other salts with a bivalent cation) prevents this injurious action.

In order to explain the latter result and the antagonistic effect of $CaCl_2$, the writer suggested in 1905[2] that the NaCl, in a sufficiently high concentration, rendered the membrane of the egg which was normally impermeable to NaCl (and other salts) permeable for salts, and that the addition of $CaCl_2$ prevented this injurious effect of NaCl and preserved the normal imperme-

[1] *Amer. Journ. of Physiol.*, iii, p. 327, 1900; vi, p. 411, 1902; *Arch. f. d. ges. Physiol.*, lxxxviii, p. 68, 1901; Loeb and Gies: *ibid.*, xciii, p. 246, 1902.

[2] *Arch. f. d. ges. Physiol.*, cvii, p. 252, 1905.

ability of the membrane.[3] The following simple experiment seems to support this view. If we put the eggs of *Fundulus* (about three days after fertilization) into a test-tube filled with a 3 M solution of NaCl, the eggs will float at first but sink after about three to six hours. In a $\frac{10}{8}$ M solution of $CaCl_2$ they will float for about half an hour. But if we put the eggs into a mixture of 50 cc. 3 M NaCl + 1 cc. $\frac{10}{8}$ M $CaCl_2$ they will float three days or more.[4] The interpretation of this experiment seems to be as follows: The membrane of the egg of *Fundulus* is impermeable for water as well as for salts, and since the interior of the egg contains a liquid of a considerably lower specific gravity than that of a 3 M solution of NaCl or of a $\frac{10}{8}$ M solution of $CaCl_2$, the egg will float in such solutions and will continue to do so as long as these solutions cannot enter the egg; that is to say, as long as the membrane is entirely or nearly intact. When exposed to a 3 M solution of NaCl or to a $\frac{10}{8}$ M solution of $CaCl_2$, each one of these solutions will in a short time alter the membrane of the egg, the $CaCl_2$ more rapidly than the NaCl, so that the membrane loses its impermeability to salts. The outside solution now diffuses into the egg, whereby the specific gravity of the egg increases and it sinks. The addition of a small amount of $CaCl_2$ to the 3 M NaCl solution prevents or retards this destructive action of the salt upon the membrane.

The next question which presents itself is: How does the addition of a small amount of $CaCl_2$ inhibit the injurious action of the pure solution of NaCl upon the membrane? The writer had found that bivalent (or polyvalent) cations had an antagonistic action upon the various salts with monovalent cations, while the bivalent or polyvalent anions had no antagonistic effect, and he called attention to the analogy of this observation with the effect of ions upon the precipitation of colloids. Negatively charged colloids can be precipitated by cations but not by anions, and the precipitating efficiency of the bivalent cations is consider-

[3] A. P. Mathews suggested in the same year the opposite view; namely, that the membrane is impermeable for a pure NaCl solution, while the addition of $CaCl_2$ renders it permeable. He explains the death of the egg in a pure NaCl solution as due to the loss of water on the part of the egg caused by the hypertonic character of the solution (A. P. Mathews: *Amer. Journ. of Physiol.*, xii, p. 419, 1905). As a matter of fact, however, a $\frac{M}{2}$ solution of NaCl is not hypertonic for the egg of *Fundulus*; and, moreover, the membrane of the egg is impermeable for water.

[4] Loeb: *Biochem. Zeitschr.*, xlvii, p. 127, 1912.

ably greater than that of the monovalent cations. We might then expect that the salts influence a colloid in the egg membrane, and this inference seems to be in agreement with all the facts.

A further tentative assumption was that the two oppositely charged ions influence the colloid of the membrane in an opposite sense. If, therefore, a trace of a salt with a bivalent metal like $CaCl_2$ renders a salt with a monovalent metal like NaCl harmless, then on this assumption the injurious action of NaCl was due to the Cl ion, and the corrective effect to the Ca ion. Such an assumption was made by the writer as well as by A. P. Mathews.[5] But there were facts which it was impossible to reconcile with such a view, as, for instance, that there was some antagonism between $SrCl_2$ and $MgCl_2$.[6] In each of these salts the effect of the cations prevails to such an extent over that of the anion that their antagonism can not be explained on the assumption that it is based on the action of the oppositely charged ions. This and other facts induced the writer to question the correctness of the view that antagonistic salt action is due to an antagonism between oppositely charged ions, and he has recently been able to show that the toxicity of NaBr, Na_2SO_4, $NaNO_3$, and other sodium salts for the adult fish of *Fundulus* can be abolished through the addition of NaCl, and other chlorides, but not by other sodium salts.[7] In this case, there could be no doubt that the toxic effects of certain anions could be counteracted through the addition of another anion; namely, Cl. It was also found that this effect was specific, since no other anion but Cl acted in this way.

This and similar facts suggested to the writer a different explanation of antagonistic salt action, which may be briefly designated as the idea that the mixture of $NaCl + KCl + CaCl_2$, in the right proportion and concentration, has a specific membrane-forming or membrane-preserving effect upon all cells; while solutions different from this mixture have a destructive effect which is the higher, the higher its concentration and the more the solution deviates from the mixture $NaCl + KCl + CaCl_2$.

However, these experiments had not been made on the eggs of *Fundulus*, which are the most favorable object for experiments on

[5] Loeb: *Amer. Journ. of Physiol.*, vi, p. 411, 1902; Mathews: *ibid*, xi, p. 455, 1904.

[6] Loeb: *loc. cit.*

[7] *Biochem. Zeitschr.*, xliii, p. 181, 1912.

the antagonistic action of salts. It seemed, therefore, necessary to find out whether there is any evidence in support of the view that antagonistic salt action in the case of the eggs of Fundulus is due to an antagonism between oppositely charged ions.

II.

In the new experiments we shall have to make use of a determination of that concentration of the chlorides of the alkaline metals and the metals of the alkaline earths which is just sufficient to prevent the formation of an embryo. Distilled water is, as we stated, harmless for these eggs and so are the solutions of any salt below a certain limit; that means in such solutions all the eggs can form embryos. Above that limit, the percentage of eggs which can form embryos becomes smaller and smaller until finally a concentration is reached at which no egg is able to form an embryo. We call this limit the toxic concentration.

TABLE I.

Toxic concentrations of chlorides.

LiCl	$>\frac{6}{32}$ M $<\frac{7}{32}$ M	$BeCl_2$	? * M
NaCl	$\frac{16}{32}$ M	$MgCl_2$	$\frac{10}{32}$ M
KCl	$>\frac{22}{32}$ M $<\frac{24}{32}$ M	$CaCl_2$	$>\frac{5}{32}$ M $<\frac{6}{32}$ M
RbCl	$>\frac{24}{32}$ M $<\frac{28}{32}$ M	$SrCl_2$	$>\frac{12}{32}$ M $<\frac{13}{32}$ M
CsCl	$>\frac{12}{32}$ M $<\frac{16}{32}$ M	$BaCl_2$	$>\frac{7}{32}$ M $<\frac{8}{32}$ M

* $BeCl_2$ forms strongly acid solutions which kill the eggs in very low concentrations. The toxic agent in this case is, however, the acid and not the $BeCl_2$.

If the antagonistic action of two salts is due to an antagonism between the ions of opposite sign, we are compelled to assume that such an antagonism exists also between the oppositely charged ions of the same salt; *e.g.*, between Na^+ and Cl^- in the case of NaCl. On the basis of that assumption we should conclude that the two opposite ions balance each other better in a KCl or RbCl solution than in a solution of LiCl or NaCl, since Table I shows that the former are less toxic than the latter. This fact might be intelligible on the assumption that Cl is the toxic ion since K and Rb are more electropositive than Li and Na. Yet it can be shown

that the difference of toxicity between the various chlorides can not be explained on the assumption of an antagonism between the oppositely charged ions of the same salt.

The writer had shown in his previous researches that if NaCl (or any other of the chlorides of this series) reaches a toxic concentration, the addition of a trace of $CaCl_2$ (or of many other salts with a bivalent metal) may render the solution harmless. On the assumption of an antagonism between the oppositely charged ions and of the toxicity of the anion, this would mean that the toxic action of Cl is incompletely balanced by the ions of Li, Na, etc., and that the trace of Mg, Ca, etc., is required to counteract the excessive action of the Cl ions. It would, moreover, be necessary to assume that this excess of the action of Cl would become greater the higher the concentration of the solution, since with increasing concentration of a salt its toxicity increases. It would follow from this, that through the addition of $CaCl_2$ it might be possible to produce embryos in a relatively higher concentration of KCl and RbCl than of NaCl or LiCl, since the former salts are less toxic,—which in the terms of our assumption would mean better balanced in regard to the oppositely charged ions. It was first ascertained that through the addition of 1 cc. $\frac{M}{1}$ $CaCl_2$ to 50 cc. of a toxic concentration of LiCl, NaCl, etc., the maximum number of embryos can be obtained.

TABLE II.

CONCENTRATION OF SOLUTION	PERCENTAGE OF EGGS WHICH FORM EMBRYOS IN 1 cc. $\frac{M}{1}$ $CaCl_2$ + 50 cc.				
	LiCl	NaCl	KCl	RbCl	CsCl
$\frac{3}{8}$ M	100				
$\frac{4}{8}$ M	30	88			72
$\frac{5}{8}$ M	2	92	87	96	
$\frac{6}{8}$ M	0	80	21	81	43
$\frac{7}{8}$ M		89	25	70	
$\frac{8}{8}$ M		93	7	18	1
$\frac{9}{8}$ M		76	0	9	
$\frac{10}{8}$ M		64		0	
$\frac{11}{8}$ M		93			
$\frac{12}{8}$ M		51			
$\frac{13}{8}$ M					
$\frac{14}{8}$ M		14			
$\frac{15}{8}$ M		0			
$\frac{16}{8}$ M		0			

The experiment with CsCl was not repeated and must therefore be left out of consideration.

It is obvious that the maximal concentration in which eggs can still form embryos is highest in the case of NaCl, where it is $\gtrless \frac{14}{8}$M. which is enormous. (It may be mentioned incidentally that this would be inconceivable if the eggs were permeable for a mixture of NaCl + $CaCl_2$, as Mathews assumes, since the fish after hatching cannot live in a solution above $\frac{9}{8}$ or possibly $\frac{10}{8}$M of NaCl + $CaCl_2$.)

If we compare the toxic limit of these salts with and without the addition of $CaCl_2$, we find the increase due to $CaCl_2$ as follows:

TABLE III.

	TOXIC LIMIT		
	Without Ca	With Ca	Increase in per cent
LiCl	$\frac{3}{16}$ M	$\frac{6}{8}$ M	400
NaCl	$\frac{16}{32}$ M	$> \frac{14}{8}$ M	350
KCl	$\frac{22}{32}$ M	$\frac{8}{8}$ M	50
RbCl	$\frac{24}{32}$ M	$\frac{9}{8}$ M	50

If the toxicity of LiCl, NaCl, KCl and RbCl were due to an excess of the action of the Cl ion over the cation; and if the Ca ion served to counteract the effects of the Cl ion, we should expect that through the addition of Ca the toxicity of KCl and RbCl would be raised at least in the same proportion as that of NaCl or LiCl; while in reality it can only be increased to $\frac{1}{8}$ or $\frac{1}{7}$ of that proportion. This means that the assumption that the toxicity of these salts is determined by an (incomplete) antagonism between the oppositely charged ions, the Cl ion being the toxic ion, is wrong or inadequate.

III.

While there may be uncertainty as regards the question whether in the case of a toxic concentration of NaCl the toxicity is due to the Na^+ or to the Cl^- ion or to the molecule, there is less uncertainty in the case of $CaCl_2$ and the other chlorides of the alkaline earth metals. In their case the toxicity must be due to the cation or to the molecule on the basis of the following consideration. The toxic concentration of $CaCl_2$ is between $\frac{5}{32}$ and $\frac{6}{32}$ M. Since

this is less than half the toxic concentration of NaCl, KCl, or RbCl, and since the toxicity of these latter salts is diminished or annihilated through the addition of Ca, it is impossible to ascribe the high toxicity of $CaCl_2$ to anything but the cation or the molecule, even if we were willing to admit that the toxicity of NaCl were due to the Cl ion.

A similar reasoning holds for $MgCl_2$, $SrCl_2$, and $BaCl_2$. The toxic concentration of $MgCl_2$ is $\frac{10}{32}$ M. We shall see later that even a $\frac{10}{8}$ M NaCl solution can be antagonized by the addition of 4 cc. $\frac{M}{1}$ $MgCl_2$, and we are certain that the antagonistic action in this case is due to the Mg. Hence, if we find that a pure $\frac{10}{32}$ M solution of $MgCl_2$, is already toxic, we can say that any toxic effect the Cl ions might have in this solution would be more than compensated by the Mg ions present (if we assume temporarily that the two ions are mutually antagonistic). Hence the toxicity of a $\frac{10}{32}$ M $MgCl_2$ solution cannot be due to the Cl ion, but must be due to the Mg ion or the $MgCl_2$ molecule. A similar reasoning applies to $SrCl_2$ and $BaCl_2$.

These salts with a bivalent cation furnish us, therefore, with a safer basis for the investigation of the question whether the antagonism between two salts exists necessarily between two ions of opposite charge than do the salts with a monovalent cation, like NaCl, where we cannot tell with certainty which of the two ions is the toxic one. If the antagonism between two salts were necessarily due to an antagonism between oppositely charged ions and if the antagonistic salt action followed exclusively the laws of the precipitation of colloids, we should expect that where the cation is the toxic agent it should be more efficiently antagonized by a salt with a polyvalent anion than with a monovalent anion, since the precipitating efficiency of ions upon colloids increases rapidly with the valency of the ion. If, therefore, the toxicity of a pure $\frac{10}{32}$ M solution of $MgCl_2$ is due to the Mg ion, it should be easier to antagonize this solution with sodium sulphate or sodium citrate than with sodium chloride. The experiments of Table IV show, however, that there is hardly any difference between the antagonistic effect of these three salts. The method of procedure is as follows: The toxic concentration of $MgCl_2$ used was a $\frac{5}{16}$ M solution in which the newly fertilized eggs of *Fundulus* can, as a rule, no longer develop. As antagonistic salts, sodium chloride, sodium acetate,

sodium sulphate, and sodium citrate were used. The $\frac{5}{16}$ M $MgCl_2$ solution was made up with the antagonistic salt solution (instead of with distilled water), so that the concentration of $MgCl_2$ was the same in each solution, namely, $\frac{5}{16}$ M; while 50 cc. of this solution contained varying quantities of the antagonistic salt.

TABLE IV.

ANTAGONISTIC SALT	PERCENTAGE OF EGGS WHICH FORMED EMBRYOS IN 50 CC. $\frac{5}{16}$ M $MgCl_2$ CONTAINING IN ADDITION														
	0	0.5	1.0	1.5	2.0	2.5	3.0	4.0	5.0	6.0	8.0	10.0	12.0	15.0	20.0
	CC. OF ANTAGONISTIC SALT														
$\frac{M}{1}$ NaCl	0	5	21	20	36	45	46	52	70	70	81	59	50	77	
$\frac{M}{1}$ Na acetate	0	18	23	43	32	42	39	30	60	45	53	40	34	46	
$\frac{M}{2}$ Na_2SO_4	0	14	30		46		70	64		76	71	75	80	87	73
$\frac{M}{2}$ Na citrate	0	12	15		50		63	77		66	54	57	28	25	4

The difference in the antagonistic action of these four salts observed in Table IV is within the limits of error and individual variation. This proves that the antagonization of the toxic effects of a cation is in this case not caused by an anion but by a cation or by the molecule. This fact has an important bearing on the writer's former observation that a toxic concentration of NaCl could be antagonized by bivalent or polyvalent cations, but not by bivalent or polyvalent anions. This appeared at that time as an argument in favor of the idea that the toxic action of NaCl was due to the anion. We now find that in a case in which the toxic agent is surely not an anion, but either a cation (Mg) or a molecule, the same law prevails; namely, that the valency or nature of the anion has practically no influence upon the antagonistic efficiency of a salt. We can, therefore, not use the inefficiency of anions in a case of antagonistic salt action as an argument in favor of the assumption that the toxic agency in such a case must be an anion. If the antagonistic action of the sodium salts against $MgCl_2$ was not due to the anion, it must have been due to the cation, namely, Na, or to the molecule.

We can show that the toxic limit of a $MgCl_2$ solution can be raised by other chlorides much more efficiently than by NaCl. We have seen that in a $\frac{10}{32}$ M solution of $MgCl_2$ as a rule the newly fertilized egg of *Fundulus* does not live long enough to form an embryo. The

question presented itself: By which type of salts can we raise the toxic limit of $MgCl_2$ more effectively, by Na salts with polyvalent anions, or by chlorides of different metals? The experiments showed that the latter was the case.

Newly fertilized eggs of *Fundulus* were put into solutions each containing 50 cc. $\frac{10}{32}$, $\frac{11}{32}$, and $\frac{12}{32}$ M $MgCl_2$ containing varying amounts of sodium citrate or of KCl. It was found that the addition of sodium citrate did not allow the eggs to develop in a $MgCl_2$ solution above $\frac{10}{32}$ M, while with the addition of KCl some eggs could still develop in $\frac{12}{32}$ M $MgCl_2$.

TABLE V.

Percentage of embryos formed in 50 cc. $MgCl_2$ *in which were contained varying quantities of* KCl *or of sodium citrate.*

CONCENTRATION OF $MgCl_2$	0	0.5	1.0	2.0	4.0	6.0	0	2	4	8
	cc. 2½ M KCl						cc. $\frac{M}{2}$ SODIUM CITRATE			
$\frac{10}{32}$ M	0	18	30	55	8	0	0	2	9	13
$\frac{11}{32}$ M	0	1	18	32	5	0	0	0	0	0
$\frac{12}{32}$ M	0	1	0	0	0	0	0	0	0	0

The slight antagonistic effect of sodium citrate in Table V was due to the Na ion and not to the citrate ion, since we have seen in Table IV that NaCl acts in the same way. It would be of the greatest theoretical importance to find other cases in which a cation is both the toxic as well as the antagonistic agent. It would deprive us of the excuse for arguing that because Ca antagonizes NaCl, the toxicity of the latter must be due to the anion. This proof will be given in the next section.

IV.

In one of his earliest papers on salt action, the writer had already shown that a toxic solution of $CaCl_2$ could be antagonized by KCl and NH_4Cl, but not by NaCl and LiCl. Since we now are certain that the toxicity of $CaCl_2$ is not due to the anion but to the cation, the question whether cations can antagonize the toxic action of other cations became of interest. For this reason, experiments like those of Table V were carried on with toxic solutions of $MgCl_2$, $CaCl_2$, $SrCl_2$, and $BaCl_2$. It was found that a $\frac{5}{16}$ M solution of

$MgCl_2$ could be antagonized by NaCl, KCl, RbCl, CsCl, and NH_4Cl, but not by LiCl; $CaCl_2$ could be antagonized by KCl, RbCl, CsCl, and NH_4Cl, but not by LiCl and NaCl; $SrCl_2$ could be antagonized by NaCl, KCl, and RbCl, but not by LiCl; and $BaCl_2$ by LiCl, NaCl, KCl, RbCl, CsCl, and NH_4Cl. The results are set forth in Tables VI to IX.

TABLE VI.

ANTAGONISTIC SALT	PERCENTAGE OF EGGS FORMING EMBRYOS IN 50 cc. $\frac{5}{16}$ M $MgCl_2$ CONTAINING IN ADDITION					
	0	0.5	1.0	2.0	4.0	6.0 cc. 2½ M
	ANTAGONISTIC SALT					
LiCl	0	0	0	0	0	0
NaCl	0	13	36	40	41	22
KCl		10	60	62	46	19
RbCl		40	41	30	0	0
CsCl		18	27	10	0	0
NH_4Cl		10	44	6	0	0

TABLE VII.

ANTAGONISTIC SALT	PERCENTAGE OF EGGS FORMING EMBRYOS IN 50 cc. $\frac{5}{16}$ M $CaCl_2$ CONTAINING IN ADDITION					
	0	0.5	1.0	2.0	4.0	6.0 cc. 2½ M
	ANTAGONISTIC SALT					
LiCl	0	0	0	0	0	0
NaCl	0	0	0	0	0	0
KCl	0	15	9	21	37	45
RbCl		5	8	11	33	18
CsCl		0	12	15	6	7
NH_4Cl		0	0	3	5	9

TABLE VIII.

ANTAGONISTIC SALT	PERCENTAGE OF EGGS FORMING EMBRYOS IN 50 cc. ½ M $SrCl_2$ CONTAINING IN ADDITION					
	0	0.5	1.0	2.0	4.0	6.0 cc. 2½ M
	ANTAGONISTIC SALT					
LiCl	0	0	0	0	0	0
NaCl		0	3	3	12	10
KCl		1	10	20	18	4
RbCl		0	4	7	2	0

TABLE IX.

ANTAGONISTIC SALT	PERCENTAGE OF EGGS FORMING EMBRYOS IN 50 CC. $\frac{5}{2}$ M $BaCl_2$ CONTAINING IN ADDITION ANTAGONISTIC SALT					
	0	0.5	1.0	2.0	4.0	6.0 cc. $2\frac{1}{2}$ M
LiCl	0	8	12	22	4	0
NaCl		0	0	8	9	0
KCl		5	3	11	10	22
RbCl		3	0	13	18	5
CsCl		4	4	28	25	1
NH_4Cl		36	60	57	5	0

The very powerful action of NH_4Cl against $BaCl_2$ was confirmed in other experiments. They suggest a specific action of the various salts.

In view of these results it is no longer surprising that we should also find some antagonism between two salts with bivalent cations and the same anion. Such observations would remain a riddle if we considered antagonistic salt action as necessarily due to oppositely charged ions. It is difficult to show that the toxic action of $CaCl_2$ is antagonized as well by KCl as by potassium citrate or sulphate, on account of the precipitate formed. The experiments with $MgCl_2$ given in Table IV, however, serve this purpose.

V.

The results of this paper harmonize with the conclusions at which the writer arrived in his more recent studies on the same subject.[8] If it is true that the injurious action of a single salt in a sufficiently high concentration consists in an alteration of the membrane, the antagonistic salt action consists in a diminution or prevention of that alteration. In a mixture of NaCl + KCl and $CaCl_2$ in the right concentration the membrane lasts the longest and we may understand that the egg can resist a higher concentration of a mixture of NaCl + $CaCl_2$ than of KCl + $CaCl_2$ or RbCl + $CaCl_2$ or of LiCl + $CaCl_2$, for the simple reason that NaCl + $CaCl_2$, in the proportion of 50 cc. NaCl to 1 cc. or less $CaCl_2$, comes nearer the optimal mix-

[8] *Biochem. Zeitschr.*, lxvi, p. 270, 1914.

ture $NaCl + CaCl_2 + KCl$ in that proportion in which these salts exist in the sea water, than any other combination of two salts in the same proportion.

On the basis of this argument we should expect that $CaCl_2$ is able to antagonize a higher concentration of NaCl than either $MgCl_2$ or $SrCl_2$ or $BaCl_2$. Experiments made to establish the highest concentration of the combination of NaCl with the chlorides of Mg, Ca, Sr, and Ba, in which newly fertilized eggs are able to form an embryo gave the following result.

TABLE X.

Highest concentration of NaCl *solutions in which the newly fertilized eggs of Fundulus can still form an embryo.*

50 cc. $\frac{10}{8}$ M NaCl + 4 cc. $\frac{M}{1}$ $MgCl_2$
50 cc. $\frac{14}{8}$ M NaCl + 1 cc. $\frac{M}{1}$ $CaCl_2$
50 cc. $\frac{14}{8}$ M NaCl + 1 cc. $\frac{M}{1}$ $SrCl_2$
50 cc. $\frac{7}{8}$ M NaCl + 1 cc. $\frac{M}{1}$ $BaCl_2$

The difference is striking and easily understood on the assumption that the combination $NaCl + CaCl_2$ is a nearer approach to the mixture $NaCl + KCl + CaCl_2$ than any of the other combinations mentioned in the table.

The question may then be asked: Why can we substitute even to some extent Mg or Sr or Ba (or Pb and Zn) for Ca? The answer must be that all these metals must have one property in common with Ca, which is of importance for the preservation of the membrane, and this property may well be the formation of a membrane of precipitation at the surface of the egg, as Traube's theory would demand. It is very likely that the same membrane-forming substance of the cell which is precipitated by Ca is also precipitated by Mg, Sr, Ba, and other bivalent metals. That these metals are not as satisfactory as $CaCl_2$ may be due to the fact that the calcium precipitate has certain physical properties which are not shared by the precipitates with other metals.

The writer had called attention to the fact that the difference in the action of Na, K, and Ca upon the absorption of water in muscle resembled the influence of the same ions on the absorption of water by soaps;[9] and Hansteen Cranner[10] has shown that the

[9] Loeb: *Arch. f. d. ges. Physiol.*, lxxv, p. 303, 1899.
[10] *Jahrbücher f. wissensch. Botanik*, liii, p. 536, 1914.

same difference exists for the absorption of water by the roots of plants as well as by the isolated membranes of plant cells. Ca forms solid soaps, and Robertson has pointed out that the fact that so little Ca is needed for its antagonistic action harmonizes with the assumption that its action depends upon the formation of a precipitate.[11]

A complete theory of antagonistic salt action may also have to consider the fact that the cell walls consist of more than one chemical compound. As far as plant cells are concerned we know that this is true, since they contain aside from cellulose and pectine substances also fatty acids (not lecithin), as Hansteen Cranner[12] has recently shown.

The experiments of Beutner and the writer on the origin of electrical phenomena in animals have also led to the conclusion that the surface of the cell contains higher fatty acids or other water-immiscible substances.[13] The bulk of the cell wall must be of a different chemical character. The fact that the importance of the membrane of animal cells has so long been underestimated has left a gap in our knowledge of this organ, and this prevents us from formulating a complete theory of antagonistic salt action.

Whatever this theory may be in detail, we may be sure that the facts of antagonistic salt action cannot be expressed by the assumption that it is based upon the action of oppositely charged ions.

SUMMARY.

1. The main object of this paper is an investigation of the question whether antagonistic salt action is based on an antagonism between oppositely charged ions. It is shown that this assumption leads to difficulties if applied to the antagonization of a toxic salt with a monovalent cation by a salt with a bivalent cation.

2. It is shown that for the toxic concentrations of $MgCl_2$, $CaCl_2$, $SrCl_2$, and $BaCl_2$ the cation is the toxic agency; and that, nevertheless, the efficiency of their antagonists is determined by the cation and not by the anion.

[11] T. B. Robertson: *Ergeb. d. Physiol.*, x, p. 216, 1910.

[12] *Loc. cit.*

[13] Loeb and Beutner: *Biochem. Zeitschr.*, li, p. 288, 1913; lix, p. 195, 1914.

THE CATALYTIC REACTIONS OF BLOOD.

I. A STUDY OF SOME OF THE FACTORS INVOLVED IN THE BENZIDINE TEST FOR OCCULT BLOOD.

By W. G. LYLE, L. J. CURTMAN and J. T. W. MARSHALL.

(*From the Harriman Research Laboratory, Roosevelt Hospital, New York.*)

(Received for publication, July 17, 1914.)

The benzidine-hydrogen dioxide test for minute quantities of blood, introduced by Adler, is described in nearly every textbook on physiological chemistry. Despite its general use, nothing very definite appears to be known concerning the conditions under which this test may be relied upon to give consistent results. The lack of uniformity in the procedures which are used in clinical practice indicated the need for an investigation of the various factors involved in this test, with a view to establishing the best conditions for carrying out the reaction. Some preliminary work with acetic acid solutions of benzidine[1] showed that the reaction was influenced by the following factors: the concentrations of (a) benzidine; (b) acetic acid; (c) hydrogen dioxide; (d) the quality of the reagents; also (e) the *order* of addition of the reagents; (f) the age of the benzidine solution.

We have found also that, in order to get uniform results, the most scrupulous care must be exercised in cleaning all the glassware (burettes, flasks, test-tubes, etc.) employed in the preparation and use of the reagents. Treatment with chromic acid, followed by thorough scrubbing with soap solution and rinsing with redistilled water, answered this purpose.

[1] Although alcoholic solutions are recommended in textbooks, preliminary experiments showed them to be unsuitable, for the reason that satisfactory blanks could not be obtained, even when the reagent was prepared with alcohol redistilled from glass. As we found the simpler aldehydes to give strong tests, this failure to obtain negative controls may be due to small quantities of aldehyde, either present in the alcohol or produced by the action of the hydrogen dioxide. Alcoholic solutions were, therefore, eliminated in this investigation.

An extensive series of preliminary experiments showed that the ordinary technique, when applied to blood at high dilution, was capable of yielding results varying from a decidedly positive test to a negative one, the results depending upon the relations existing between the concentrations of benzidine, acetic acid, and hydrogen dioxide. It thus became evident that the most satisfactory reagent was to be found only by testing a large number of combinations in which the concentrations of the above mentioned substances were systematically varied. In this connection it may be well to mention that with very low concentrations of hydrogen dioxide and acetic acid, positive tests were obtained with tap water, laboratory distilled water, and even with the latter redistilled from glass.[2] Throughout this investigation it was necessary, therefore, to control every test by one or more blanks.

The method recommended in most textbooks for making the benzidine test consists in adding to 1 cc. of a saturated acetic acid solution of benzidine, 1 cc. of the suspected liquid, and 1 cc. of 3 per cent hydrogen dioxide. This procedure is open to two objections: first, the solubility of benzidine in acetic acid varies greatly with the temperature; second, such a solution, freshly prepared at room temperature, when diluted 1:3 with water gives a heavy crystalline precipitate which seriously interferes with the test. In our experimental work with solutions of various concentrations of benzidine, it was imperative, therefore, to rule out those which crystallized under the conditions of the test.

Since crystallization may be prevented by an excess of acetic acid, the following experiments were made to determine the minimum amount of acid required for this purpose. A rough determination showed that at 18° the solubility of benzidine in glacial acetic acid is 16 grams in 100 cc. Accordingly, a 15 per cent solution was prepared by dissolving 3.75 grams of Kahlbaum's benzidine in about 23 cc. of glacial acetic acid, with slight heating,[3] and subsequently making up the volume to 25 cc. with acetic

[2] In the preparation of the redistilled water used throughout this investigation, a still of which all the parts were glass was employed.

[3] It was found that after greater heating, benzidine solutions require less acetic acid to prevent crystallization. Prolonged heating, however, is objectionable for reasons given later.

acid. If to 1 cc. of this solution, 1 cc. of water be added, crystallization sets in; if 1.5 cc. of water be added, 0.1 cc. of acetic acid must be added to prevent crystallization; if 2.5 cc. of water be added, 0.25 cc. of acetic acid is required. The series of experiments made to determine the quantity of acetic acid necessary to hold various concentrations of benzidine in solution at 18° gave the following results:

Benzidine *per cent*	Acetic acid required to prevent crystallization at 18° *per cent*
8	50[4]
6	40
3	25

In our investigation we decided to experiment with reagents containing benzidine in concentrations of 6, 4, 2, and 1 per cent. The amount of acetic acid required to prevent crystallization in solutions of these concentrations was then calculated from the data given above.

With each concentration of benzidine, the acetic acid was varied from the minimum required to prevent crystallization to a considerable excess. Again, with each concentration of acetic acid, the strength of the hydrogen dioxide was varied over a wide range. This gave in all about seventy different combinations. The range of benzidine, acetic acid, and hydrogen dioxide in the reagents experimented with may be seen in the following table:

SERIES	BENZIDINE	ACETIC ACID[5]	HYDROGEN DIOXIDE
	per cent	*per cent*	*per cent*
A	6	41–77	0.020–0.96
B	4	28–57	0.014–1.37
C	2	16–44	0.067–2.40
D	1	8–26	0.033–2.20

[4] All the percentages in this work were calculated on the final volume; i.e., after the addition of the solution to be tested and the dioxide. As the ordinary procedure calls for about 15 per cent benzidine solution diluted 1:3 in making the test, the final mixture contains about 5 per cent of benzidine and 31 per cent of acetic acid.

[5] Here, as elsewhere, the calculations are based on glacial acetic acid (99.5 per cent), which was used throughout the work.

As an example of the general procedure employed in the preparation and use of the reagents of various concentrations, the following may be cited. In a small Erlenmeyer flask, 2 cc.[6] of 15 per cent of benzidine solution[7] were mixed with 1.20 cc. of acetic acid and 3.20 cc. of redistilled water, making a total volume of 6.40 cc. Therefore, 2.56 cc. of this mixture contained 0.80 cc. of the original 15 per cent benzidine solution, or 0.12 gm. of benzidine. This amount (2.56 cc.) of solution was then introduced into a test-tube by means of a graduated pipette and 0.20 cc. of blood solution added. (In making the controls, 0.20 cc. of redistilled water was used in place of the blood.) Finally 0.24 cc. of 3 per cent hydrogen dioxide was added, thus making a total volume of 3 cc. This gives a reagent containing benzidine, acetic acid, and dioxide in the following proportions: benzidine (0.12 gm. in 3 cc.), 4 per cent; acetic acid (1.22 cc.[8] in 3 cc.), 40.5 per cent;[9] hydrogen dioxide (0.24 cc. 3 per cent H_2O_2=0.0072 gm.), 0.24 per cent.

The results obtained with the various reagents are summarized in the following tables:

[6] All solutions were delivered from burettes, except where otherwise stated.

[7] 100 cc. of this glacial acetic acid solution contained 15 gm. of benzidine.

[8] That this quantity of acetic acid was present may be seen from the following considerations: 15 gm. of benzidine dissolved in 92 cc. of glacial acetic acid yield 100 cc. of 15 per cent solution. Therefore, 2 cc. of this solution will contain 1.84 cc. of acetic acid. As 1.20 cc. additional acetic acid were added, the total amount present in 6.40 cc. of the mixture is 3.04 cc.; and in 2.56 cc., the quantity of the mixture taken for the test, the amount is 1.22 cc.

[9] This is the total amount of acid used in the preparation of the reagent and includes that in combination with the benzidine.

TABLE I.

Series A. 6 per cent benzidine. Blood 1:500,000 (0.2 cc. of 1:100,000 blood solution).[10]

REAGENT	ACETIC ACID	H_2O_2	BLANK	TEST
	per cent	*per cent*		
	41	<0.24	+	
Aα4[11]	41	0.24	±	
Aα5	41	0.48	−	Good.
Aα6	41	0.96	−	Fades too quickly.
Aβ3	45	0.18	+	
Aβ4	45	0.48	−	Good.
	45	>0.48	−	Fades too quickly.
	57	<0.18	+	
Aγ3	57	0.18	±	
Aγ4	57	0.48	−	Fades quickly.
	57	>0.48	−	Fades quickly.
	77	<0.18	+	
Aδ3	77	0.18	−	Fades quickly.
Aδ4	77	0.48	−	Fades quickly; better than Aδ3.

Comments. An examination of Table I shows that as the concentration of acetic acid is increased, less hydrogen dioxide is required to give a satisfactory blank and that with these higher concentrations of acid, the color fades more rapidly. Reagents Aα5, Aβ4, Aγ4, and Aδ4 were found to be the best of their respective groups. On comparing these reagents simultaneously, Aα5 proved the most satisfactory of the entire series.

[10] The blood solutions employed throughout this work were prepared as follows: Blood of normal haemoglobin content was taken from the finger with a blood pipette, the capillary portion of which had a determined capacity of 58 mgm. of water. This quantity of blood was immediately diluted to 58 cc. with redistilled water, giving a 1:1000 blood solution. From this, by suitable dilution, solutions of lower concentrations were prepared. Although the ordinary technique calls for 1 cc. of suspected fluid in a total volume of 3 cc., we preferred in our work to employ a smaller volume of correspondingly stronger blood solution; thus the 0.2 cc. of blood solution 1:100,000 is equivalent to 1 cc. (the amount of suspected solution employed in the ordinary procedure) of blood 1:500,000.

[11] In the notation employed throughout this work the capital letters A, B, C, etc., refer to the per cent of benzidine, the Greek letters to the per cent of acetic acid, while the numerals refer to the per cent of H_2O_2.

TABLE II.

Series B. 4 per cent benzidine. Blood 1:500,000.

REAGENT	ACETIC ACID	H_2O_2	BLANK	TEST
	per cent	*per cent*		
	28	<0.28	+	
Bα5	28	0.28	+	
Bα5½	28	0.50	+	
Bα6	28	0.60	−	Good.
	28	>0.60	−	Fades quickly.
Bβ3	32	0.24	+	
Bβ4	32	0.64	−	Fair.
	32	>0.64	−	Fades quickly.
Bγ2	41	0.24	+	Good.
Bγ3	41	0.64	−	Fades too quickly.
	41	>0.64	−	Fades too quickly.
Bδ2	57	0.24	−	Faint.
	57	>0.64	−	Fades too quickly.

Comments. The above table shows that Bα6, Bβ4, Bγ2, and Bδ2 are the best of the B series, although Bγ2 did not give consistently good blanks. Another set of experiments in which the above four reagents were compared indicated that Bα6 was the best of the B series.

TABLE III.

Series C. 2 per cent benzidine. Blood 1:500,000.

REAGENT	ACETIC ACID	H_2O_2	BLANK	TEST
	per cent	*per cent*		
Cα3	16	0.26	+	
Cα3½	16	0.40	±	Good.
Cα4	16	0.54	−	Good.
Cα5	16	0.80	−	Good.
Cβ2½	20	0.40	±?	Good.
Cβ3	20	0.54	−	Good.
Cβ4	20	0.80	−	Good.
	28	<0.40	+	
Cγ2½	28	0.40	−	Fair.
Cγ3	28	0.80	−	Good but fades in 5 min.
	28	>0.80	−	Fades too quickly.
Cδ2	44	0.26	±	
Cδ2½	44	0.40	−	Fair; fades in 7 min.
Cδ2¾	44	0.54	−	Fair; fades in 7 min.
Cδ3	44	0.80	−	Fades very quickly; gone in 5 min.

This series afforded so many satisfactory reagents that it was thought advisable to perform another set of experiments in which only the best of the series were compared.

The results are given in Table IV.

TABLE IV.

Series C. 2 per cent benzidine. Blood 1:500,000.

REAGENT	ACETIC ACID	H_2O_2	BLANK	TEST
	per cent	*per cent*		
$C\alpha 3\frac{1}{2}$	16	0.40	± in 4 min.	Good green in 5 min.
$C\alpha 4$	16	0.54	—	Blue > α $3\frac{1}{2}$.
$C\beta 2\frac{1}{2}$	20	0.40	±? in 4 min.	Good green in 5 min.
$C\beta 3$	20	0.54	—	>β $2\frac{1}{2}$ in 6 min.
$C\gamma 2\frac{1}{2}$	28	0.40	—	Faint green in 4 min.; fading in 7 min.
$C\gamma 2\frac{3}{4}$	28	0.54	—	Fair green in 4 min. >$C\gamma 2\frac{1}{2}$ in 6 min.
$C\delta 2\frac{1}{2}$	44	0.40	—	Fair green in 4 min.; fades in 7 min.
$C\delta 2\frac{3}{4}$	44	0.54	—	Fair green in 5 min.; fading in 5 min.

Comments. With concentrations of acetic acid of 20 per cent or over, the test does not develop strongly, and, furthermore, with the higher percentages of acid, the color fades very rapidly. The choice therefore lies in the α series. Since $C\alpha 3\frac{1}{2}$ gave suspicious blanks, $C\alpha 4$ seems the most suitable reagent of the C series.

TABLE V.

Series D. 1 per cent benzidine. Blood 1:500,000.

REAGENT	ACETIC ACID	H_2O_2	BLANK	TEST
	per cent	*per cent*		
	8	<0.40	+	
D$\alpha 4\frac{1}{2}$	8	0.40	—	Good.
D$\alpha 5$	8	0.54	—	Good.
D$\alpha 6$	8	0.80	—	Good but fades more quickly than D$\alpha 5$.
D$\beta 2$	10	0.13	+ 6 min.	
D$\beta 3$[12]	10	0.27	± 8 min.	Good.
D$\beta 4$	10	0.54	± 8 min.	Strong; fading in 6 min.
D$\beta 5$	10	0.80	—	Fades too fast.
D$\gamma 1\frac{1}{2}$	16	0.07	—	Very faint; fading in 8 min.
D$\gamma 2$	16	0.13	—	Very faint; fading in 8 min.
D$\gamma 3$	16	0.40	—	Fair; fading in 6 min.
D$\gamma 4$	16	1.20	—	Strong; fading in 5 min.
	26	0.07–2.20	—	
D$\delta 2\frac{1}{2}$	26	0.27	—	Very faint; fades quickly.
D$\delta 3$	26	0.53	—	Faint; fades too quickly.
D$\delta 3\frac{1}{2}$[13]	26	1.00	—	Good; fades too quickly.

To determine the best reagent of the D series, further experiments were made in which only the best of the series D[14] were compared. The results are summarized as follows:

TABLE VI.

Series D. 1 per cent benzidine. Blood 1:500,000.

REAGENT	ACETIC ACID	H_2O_2	TEST AT THE END OF	
			4 min.	7 min.
	per cent	*per cent*		
D$\alpha 4\frac{1}{2}$	8	0.40	Blue < $\alpha 5$	Blue < $\alpha 5$.
D$\alpha 5$	8	0.54	Strong blue	Strong blue.
D$\beta 3$	10	0.27	Blue < $\alpha 4\frac{1}{2}$	Blue < $\alpha 4\frac{1}{2}$.

The above results show that with blood $2:10^6$, D$\alpha 5$ gave the best test. When, however, blood of concentration $2:10^7$ was used,

[12] With blood 10^{-6} D$\beta 3$ gives a decidedly better test than D$\beta 4$.

[13] With blood 10^{-6} none of the δ series gave a satisfactory test.

[14] The tests with the γ and δ series faded so rapidly that these reagents were eliminated in subsequent experiments.

it was found that, while D$\alpha 4\frac{1}{2}$ developed a fair blue in 6 minutes, D$\alpha 5$ gave only the faintest test. D$\alpha 4\frac{1}{2}$ was therefore considered the best of the D series.

General comments and conclusions. Throughout this investigation it was found that quantities of acetic acid in large excess over those required to prevent crystallization interfered with the delicacy of the test by increasing the speed of fading of the blue color. The most suitable reagents in the entire series were found among the α concentrations of acetic acid; *i.e.*, those containing the minimum quantities of acid. It was also observed that an excess of hydrogen dioxide diminished the sensitiveness of the reagent by rapidly bleaching the blue color. On the other hand, with a relatively small quantity of dioxide, satisfactory blanks were not obtained, and, in addition, with blood, the color developed very slowly. Of the A series A$\alpha 5$ proved the most satisfactory reagent. B$\alpha 6$ was the best of the B series. C$\alpha 4$ seemed the most desirable of the C series and D$\alpha 4$ the best of series D.[15]

Comparison of the best reagents. With a view to determining the most sensitive reagent, the best of each of the four series was selected and these were compared. The results of a number of experiments, with these reagents with blood solutions of various concentrations, showed that with blood $2:10^6$, reagent C$\alpha 4$ was the most satisfactory. With blood $1:10^6$, C$\alpha 4$ developed a good blue, but this color faded more quickly than that given by D$\alpha 4\frac{1}{2}$. When blood $5:10^7$ was used, C$\alpha 4$ gave only a faint green, while D$\alpha 4\frac{1}{2}$ gave a fair blue. D$\alpha 4\frac{1}{2}$ seems, therefore, to be a more sensitive reagent than C$\alpha 4$. Reagents A$\alpha 5$ and B$\alpha 6$, containing respectively 6 and 4 per cent benzidine, while fairly sensitive, do not compare favorably with the C and D reagents for the following reasons:[16]

[15] All the reagents selected gave perfect blanks in the numerous experiments carried out at room temperature, which varied from 18–20°. When, however, towards the end of this investigation, the temperature rose to 24°, doubtful controls were obtained. This difficulty was obviated by the addition of 0.1–0.2 cc. of glacial acetic acid to the amount of reagent required for each test. Under these conditions no appreciable difference in the sensitiveness of the reagents towards blood was observed.

[16] In our study of inhibitors, an investigation which is in progress at the present time in this laboratory, we found that with blood solutions of fairly high concentration to which tannic acid had been added, or with urines

a. While giving strong tests with blood solutions of moderate concentration, they do not respond to highly dilute solutions.

b. The A and B reagents (containing 6 and 4 per cent of benzidine) are of a decided yellow-brown color which renders the detection of a slight blue tint very difficult.

c. When freshly prepared, reagents A and B do not possess the latitude of the more dilute benzidine solutions, a slight lowering of temperature or a relatively small increase in the percentage of water causing crystallization.

d. They are more affected by a decrease in concentration of hydrogen dioxide, such as may be caused by the accidental addition of a slight excess of water; thus, if to a volume of 3 cc. of complete reagent 0.2 cc. of water be added, a doubtful and in some cases a positive test is obtained.

e. They are decidedly more costly, as they contain more benzidine.

Preparation and use of the reagent. For routine work we suggest that the reagent be prepared in the following manner: To 4.33 cc. of glacial acetic acid, contained in a small Erlenmeyer flask and warmed to 50°, add 0.5 gram of benzidine.[17] Heat the flask for 8–10 minutes[18] in water maintained at 50°. To the solution add 19 cc. of water redistilled from glass.

Into a perfectly clean, dry test-tube, introduce 1.4 cc. of the above benzidine solution, add 0.2 cc. of water or glacial acetic acid,[19] then 1 cc. of the fluid to be tested,[20] and finally 0.4 cc. of 3 per cent hydrogen dioxide freshly prepared from perhydrol. This procedure gives 3 cc. of a solution containing 1 per cent of benzidine, 0.4 per cent of hydrogen dioxide, and 1 cc. of suspected liquid.

containing moderate quantities of blood, the stronger reagents seemed to give better tests, due probably to the increased active mass of benzidine in proportion to that of the inhibitor.

[17] The purest obtainable; we used Kahlbaum's for reasons given below.

[18] The benzidine usually dissolves before the expiration of this time, but the continued heating is advised to prevent crystallization in cold weather.

[19] In cold weather the acetic acid should be replaced by redistilled water.

[20] For a control add 1 cc. of redistilled water instead of the suspected fluid.

Order of addition of the reagents. We have found that if hydrogen dioxide be added to a solution of blood, the catalytic activity of the latter is rapidly destroyed. That this is the case is shown by the fact that if to 0.1 cc. of blood 1:1000, 0.2–0.3 cc. of 3 per cent hydrogen dioxide be added and the mixture allowed to stand for several minutes, a negative test results on the addition of benzidine, even though a fresh quantity of dioxide be subsequently added. We have evidence also that a mixture of benzidine and dioxide decomposes rapidly and loses its sensitivity, although no blue color develops. Experiments showed, however, that a mixture of blood and benzidine does not appear to deteriorate appreciably within an hour. Therefore, in making the test, the H_2O_2 must be added last; *i.e.*, to the mixture of blood and benzidine solution.

Purity of the reagents. Although both Merck's "Reagent for Blood Test" and Kahlbaum's benzidine ran low in nitrogen and contained traces of iron,—this trace of iron may account for the difficulty in obtaining perfect blanks under certain conditions,—we chose Kahlbaum's because it proved to be of more uniform purity and gave a lighter colored solution. Both Merck's and Kahlbaum's "Reagent" acetic acid gave good results, but unless redistilled the "C. P." grades did not give perfect blanks. Merck's "Perhydrol" diluted to contain 3 per cent hydrogen dioxide was used throughout this investigation. An examination of the leading brands of dioxides showed that those containing sulphates or acetanilid gave unsatisfactory results.

Age of benzidine solutions. It is generally believed that acetic acid solutions of benzidine rapidly deteriorate in sensitivity towards blood, some investigators[21] stating that with certain preparations there is a falling off of 50 per cent in twenty-four hours. We did not find such rapid deterioration in our solutions, however. A 15 per cent solution of benzidine in glacial acetic acid does not lose appreciably in sensitivity for three days; after this time the solutions become so dark that any green color resulting from the test may have been masked. However, with some 15 per cent solutions which chanced to stand exposed to the air for periods of a week or more, excellent tests were obtained with blood $1:10^6$,

[21] Ruttan and Hardisty: *Biochem. Bull.*, ii, p. 225, 1912–13.

the blanks being at the most only faintly suspicious. With the 2 per cent solution in 18 per cent acetic acid, the results given in the table show that not only is there no deterioration, but in fact an improvement with age, thus confirming the previously mentioned results obtained with a number of old solutions. The statement that benzidine solutions rapidly lose their sensitivity on standing must therefore be taken with reserve.

SUMMARY.

1. An investigation was made of the chief factors involved in the benzidine test for occult blood. Our results, with reagents covering a wide range of concentrations of benzidine, acetic acid, and hydrogen dioxide, show that the sensitivity of the test was greatly influenced by the relative concentrations of these substances.

2. Dilute solutions of benzidine are exceedingly sensitive to pure aqueous solutions of blood, and with these reagents, under the most favorable conditions, we have been able to obtain decided tests with 1 cc. of blood solution of a concentration 1 to 5 million. These reagents, however, are easily affected by inhibiting substances. By increasing the concentration of benzidine, the reagent becomes more resistant to inhibitors, but fails to respond to the more dilute aqueous solutions of blood.

3. The acetic acid serves merely to hold the benzidine in solution; an excess diminishes the delicacy of the reagent by increasing the speed of fading.

4. The hydrogen dioxide functions chiefly in two ways: first, it supplies the oxygen necessary for the reaction; second, it bleaches the blue color. Although a moderate excess of dioxide accelerates the reaction, it interferes with the delicacy of the test by increasing the speed of fading. Moreover, an excess of dioxide interferes with the reaction, both by destroying the catalytic power of the blood, and by reacting with the benzidine itself, with the formation of products which appear to have an inhibitory action. Therefore the concentration of the hydrogen dioxide should be reduced to the minimum compatible with the production of a blue color which will reach its maximum intensity within 5 or 6 minutes.

5. To obtain consistent results, water redistilled from glass should be used exclusively, and controls should invariably be made.

6. It has been shown that in making the test it is imperative that the hydrogen dioxide be added last.

7. Certain grades of hydrogen dioxide are unsuitable for the test; the best results were obtained with Merck's "Perhydrol" reagent grade. It is also essential that the benzidine and acetic acid be of the highest purity obtainable.

8. Dilute acetic acid solutions of benzidine may be kept for several days without deterioration.

We expect to publish shortly the results of an investigation of certain substances which appear to inhibit the catalytic reactions of blood, and also methods which we have devised for overcoming these difficulties in the examination of body fluids for blood.

THE ENZYMES OF ASPERGILLUS TERRICOLA.

By F. M. SCALES.

(*From the United States Department of Agriculture, Washington, D. C.*)

INTRODUCTION.

Particles of organic matter occurring in the soil under moist, aerobic and otherwise favorable conditions frequently show fungous growth, the abundance of which depends upon the completeness of the food supply. The nutrient material is made available for the fungus by its enzymes which usually produce soluble substances greatly in excess of those required by it. The excess finds its way into the soil solution where it yields its nutrient qualities to various microörganisms and plants. Many fungi, in breaking down protein material, produce considerable quantities of ammonia, which, in the form of salts, may be directly assimilated by some plants. Fungi thus play a part in maintaining the fertility of the soil by the production of nitrogenous compounds which are available for plants, and soluble carbohydrates and nitrogenous compounds which may be utilized by bacteria that have a recognized importance in the formation of plant food, and also by the formation of substances more or less humic in character.

The following investigation was undertaken under the general direction of Karl F. Kellerman, in charge of the Office of Soil Bacteriology and Plant Nutrition, to determine the scope of enzyme production of a typical soil fungus, *Aspergillus terricola*,[1] and so ascertain its possible importance, under favorable conditions, as a producer of soluble nutrient material for the soil flora.

The organism used in these experiments was isolated two years ago from a redland soil of Rome, Georgia. Since then the stock culture has been kept on Czapek's agar. This organism produces

[1] *Aspergillus terricola* was first described by Marchal (*Revue mycol.*, xv, pp. 101–3, 1893).

a luxuriant growth on potato, beef gelatin, beef agar, and Czapek's agar. The young fruiting colonies on the latter medium are a yellow ochre[2] color with a white border. When the colonies are a month old, the fruit is a raw umber[3] color. The medium is not colored and the reverse of the colony is white or colorless. The conidiophores are hyaline or light brown, continuous, rarely septate and from 100 to 280 μ in length by 6 to 8 μ in width. The walls are verruculose and about 1.5 μ thick. The vesicle is subglobose and 30 to 50 μ in diameter. Young heads have one set of sterigmata, 3 to 4 by 8 to 12 μ, while older ones frequently have two, the second set being 2 to 3 by 4 to 6 μ. The fruit head varies from 160 to 280 μ in diameter. The conidia are a light amber color, round, sometimes elliptical, thick walled, verrucose and from 4.5 to 6.5 μ in diameter. A connective can frequently be seen between the conidia. In a 2 per cent dextrose solution the conidia swell to 9 to 11 μ and germinate by sending forth a single tube from the side of the conidium; occasionally two tubes may be produced, but no branches are formed in twenty-four hours.

Cultivation of the fungus.

In most cases the fungus was grown on a modified neutral Raulin's solution containing the substance to be tested. If a carbohydrate was tested, 4.5 grams per liter of ammonium nitrate were added, or, if a nitrogenous compound, 10 grams per liter of saccharose.

This same basic solution was used with peptone (3.5 grams) and saccharose (10 grams) as the source of nitrogen and carbon. In addition to these Czapek's solution was employed as a nutrient medium.

The modified neutral Raulin's solution had the following formula:

[2] Tint No. 4, p. 326, of the *Repertoire de couleurs*, published by the French Society of Chrysanthemists, or two tones darker than the yellow ochre on Plate XV in Ridgway's *Color Standards and Color Nomenclature*, 1912.

[3] Tint No. 4, p. 301, of the *Repertoire de couleurs*, published by the French Society of Chrysanthemists, or Prout's brown on Plate XV in Ridgway's *Color Standards and Color Nomenclature*, 1912.

Distilled water	1,000	cc.
Neutral potassium tartrate	4.3	gm.
Potassium phosphate	0.4	gm.
Magnesium sulphate	0.3	gm.
Potassium sulphate	0.2	gm.
Ferrous sulphate	0.05	gm.
Test carbohydrate		
Test nitrogenous compound		

Czapek's solution was prepared according to this formula:

Distilled water	1,000	cc.
Magnesium sulphate	0.5	gm.
Dibasic potassium phosphate	1.0	gm.
Potassium chloride	0.5	gm.
Ferrous sulphate	0.01	gm.
Sodium nitrate	2.00	gm.
Saccharose	30.00	gm.

One liter flasks each containing 200 cc. of these solutions were plugged with cotton and sterilized in the autoclave at 15 pounds' pressure for half an hour. The flasks were then inoculated with conidia taken from a young growth on Czapek's agar. They were incubated at 28°C for four days. The growth on Czapek's solution was the best for the preparation of an enzyme powder, as the sterile growth was abundant and the fruit slower in maturing.

Preparation of the enzyme powder.

At the end of the growing period a slight agitation served to separate the fungous mat from the flask. The solution was poured out and about 200 cc. of distilled water were run into the flask, which was then well agitated and again drained This washing was repeated three times, when the mat was finally thrown on a filter and twice washed by filling the six inch funnel and allowing it to drain. The hyphal mass was dried first with cotton gauze and then with filter paper. It was next spread out flat and cut into squares .2 or 3 mm. on a side. The *Acetone-dauerhefe* method was used for the preparation of the enzyme powder. A 25 gram quantity of the squares of fungous material was put into a 500 cc. flask and covered with 100 cc. of acetone and agitated for ten minutes. The acetone was poured out and the fungous mass emptied onto a large filter paper which was

used to dry it. The material was returned to a flask and covered with 40 cc. of ether for three minutes; the ether was then poured off and the mass spread out on gauze to dry. The fungous tissue was quite dry and hard in half an hour, so it was put in a mill and ground to a fine powder which was then kept in a tightly sealed bottle until used.

Method of testing enzyme activity.

The hydrolytic activity of the enzyme powders was determined by adding 1 gram to 10 cc. of distilled water in a 150 cc. Erlenmeyer flask; the required quantity of the substance to be tested was then added, usually either the necessary number of cubic centimeters of a 10 per cent solution, or, if not readily soluble, the weighed amount was placed directly in the flask. In either case the volume was then made up to 50 cc. with distilled water, half a cc. of toluene added, and the flask sealed with a rubber stopper. The controls were prepared by boiling the enzyme powder in the 10 cc. of distilled water for one minute and then proceeding as in the preparation of an active solution.

A different procedure was followed in the preparation of the potato starch suspension. It was heated to rupture the starch grains and render their contents more easily available. One-half gram quantities of this substance in flasks containing 30 cc. of distilled water were heated to 100°C. in a steam sterilizer for half an hour. When the starch paste had cooled, the 10 cc. of water containing the enzyme powder were poured onto it, and any grains of the powder remaining behind were washed into the starch suspension with 10 cc. of distilled water. The antiseptic was then added and the flask sealed.

Flasks were prepared in duplicate and checked by controls in each case.

All solutions were incubated at 28°–30°C. for three days, unless otherwise stated.

GROWTH OF ASPERGILLUS TERRICOLA ON VARIOUS MEDIA AND ACTIVITY OF ENZYME PRODUCED.

Cellulose.

The source of nitrogen appears to have no influence on the growth of this organism in cellulose agar,[4] as it gave only a scant to moderate growth when either ammonium sulphate or sodium nitrate was used. When planted in a test-tube of either medium besides the surface growth a dense disk growth formed from 3 to 6 mm. below the surface. This organism is evidently a very weak cellulose destroyer, as in some cultures a month old only a very slight dissolving of the cellulose was apparent; the enzymic zone was too shallow for the demonstration of cytase by Kellerman's method.[5]

The growth on all media containing cellulose as a source of carbon was too scant for the preparation of an enzyme powder, so a powder from the growth on Raulin's peptone saccharose solution was tested. The usual amount of it was added to 50 cc. of distilled water containing a 9 cm. acid-washed filter paper and half a cc. of toluene. After a month's incubation there was no apparent destruction of the paper and the solution showed no change in reaction nor any reducing power.

Inulin.

When this carbohydrate was substituted for saccharose in the modified neutral Raulin's solution, the organism produced a thick, folded mat covering the entire surface of the liquid. An enzyme powder was prepared from this felt in the usual way and tested in 50 cc. of water containing 2 grams of inulin. This suspension was incubated for six days at the regular temperature. Five cc. of the filtered solution, to which had been added 0.2 gram of phenylhydrazine hydrochloride and 0.3 gram of crystallized sodium acetate, were immersed in boiling water. An osazone formed in

[4] I. C. McBeth and F. M. Scales: The Destruction of Cellulose by Bacteria and Filamentous Fungi, *U. S. Department of Agriculture, Bureau of Plant Industry, Bulletin No. 266*, 1913.

[5] K. F. Kellerman: Formation of Cytase by *Penicillium pinophilum*, *Bureau of Plant Industry, Circular No. 113*, pp. 29–31, 1912.

nine minutes[6] in the active solution, while the inactive one gave no precipitate in twice this time. Benedict's reagent showed that the active solution had a reducing power equivalent to 0.292 gram of invert sugar, while the control contained only 0.039 gram of this sugar.

The enzyme powder prepared from the modified neutral Raulin's solution was tested in the same way. After incubation, the active solution contained 0.042 gram, and the control 0.031 gram of invert sugar.

From these results it is seen that the enzyme from the inulin medium is over twenty times more active than that from the neutral Raulin's solution containing no inulin.

Starch.

This *Aspergillus* by its abundant sterile growth and spore production gives every indication that starch is an easily available source of carbon.

An enzyme powder was prepared from the growth on Raulin's solution containing boiled starch as the source of carbon and tested in a starch suspension in the way described. After three days' incubation the reducing power of the active solution was determined by titrating it after filtration against Benedict's reagent. The control solutions were pasty and would not pass through the filter; the starch in these was therefore precipitated by the addition of an equal volume of alcohol and a little salt. The precipitates were filtered out, the filtrates evaporated and the residues taken up with water and titrated. They contained 0.005 gram of glucose. When this quantity in the control was subtracted, the active solution contained 0.456 gram of glucose, which was equivalent to 82 per cent of the starch added.

An enzyme powder prepared from the modified Raulin's solution containing peptone and saccharose was also tested and produced 0.125 gram of glucose, which was equivalent to 22.5 per cent of the starch present.

A powder from the growth on Czapek's solution was added to

[6] Maquenne: *Compt. rend. Acad. d. sc.*, cxii, p. 799, 1891; and Mulliken: *Identification of Pure Organic Compounds*, New York, 1911, i, pp. 32–33.

50 cc. of a 2 per cent starch suspension and produced 0.190 gram of glucose, or an equivalent of 17 per cent of the starch present.

All the active solutions were tested with phenylhydrazine hydrochloride and yielded crystals of phenylglucosazone.

Saccharose.

A moderate spreading growth and fruiting was produced by this organism on Czapek's agar containing saccharose as the source of carbon.

The activity of an enzyme powder prepared from a fungous mat formed on Raulin's solution containing maltose was compared with that of a powder made from the growth on Czapek's solution containing saccharose.

The test solutions were prepared in the usual way; 50 cc. of a 5 per cent saccharose solution were used to test the powder from the growth on both the maltose and the saccharose media. At the end of the incubation period the active solutions gave a heavy yellow precipitate of osazone four minutes after starting to boil, while the control solutions gave no precipitate in fifteen minutes.

The quantity of invert sugar in the active solutions as determined with Benedict's reagent was as follows:

SOURCE OF ENZYME POWDER	INVERT SUGAR	HYDROLYSIS
	gm.	*per cent*
Maltose medium	1.25	50
Saccharose medium	2.20	88

Lactose.

This organism produced only a scant submerged growth and conidial formation in lactose solution.

One-tenth gram quantities of powder from growths in this solution were incubated in a 5 per cent solution for the usual time, but when tested with phenylhydrazine hydrochloride failed to form an osazone even after two hours' boiling. Both active and control solutions showed the same reducing power.

Maltose.

Aspergillus terricola produced an abundant growth on Raulin's solution containing maltose as the source of carbon. A powder was prepared from this fungous mat and tested in the usual way in 100 cc. of 5 per cent maltose solution. After eight days' incubation the solutions were filtered and read in a saccharimeter; the control solution gave a rotation of 41.0° Ventzke, while the active one produced a rotation of 29.7° Ventzke, which is equivalent to a 46 per cent hydrolysis. The usual quantity of an enzyme powder prepared from a growth on the regular Czapek's solution was added to 50 cc. of a 6 per cent maltose solution and incubated for three days at the usual temperature. The liquid was then filtered and read in the saccharimeter; the control solution gave a rotation of 42.1° Ventzke, and the active one a rotation of 39.7°. As the solution would have a rotation of 15.6° if completely hydrolized, the reading obtained is equivalent to a 9 per cent hydrolysis.

Glucose.

Aspergillus terricola produced a thick, much folded mat on Raulin's solution containing 5 per cent of glucose.

A powder prepared from this growth was tested in 100 cc. of 5 per cent glucose solution. After 10 days' incubation half the solution was placed in a distilling flask and about 5 cc. were distilled off. Two cc. of the distillate were tested for alcohol with a drop of benzoylchloride and a small quantity of 10 per cent sodium hydroxide, and vigorously shaken, but no ethyl benzoate was formed. Potassium bichromate and sulphuric acid also gave negative results. The experiment was repeated with the glucose solution and enzyme powder in fermentation tubes, but no gas was formed.

Tannin.

Aspergillus terricola would not grow in a sterile tap water solution containing 0.2 per cent sodium nitrate and 3 per cent tannin. It did, however, make a moderate growth with a scant production of conidia when the tannin was reduced to 0.2 per cent, which

is the same percentage that Reed[7] found to be the optimum for *Glomerella rufomaculans.*

Two cc. of the solution in which the mold had grown were diluted to 15 cc. with distilled water and then 1 cc. of 5 per cent potassium cyanide solution was added. It turned the strong pink color typical of gallic acid with this reagent, while the control showed only a trace of color. The former gave no precipitate with gelatin, but the latter formed a voluminous white one characteristic of tannin.

The activity of the enzyme in the fungous mat on the tannin solution was not determined, as there was not enough material for the preparation of a powder. An enzyme powder prepared from the growth on Czapek's medium was tested in the usual way with a 1 per cent tannin solution. After three days' incubation the active and control solutions gave a positive test with the cyanide reagent. They were filtered and diluted and their tannin content was estimated by Proctor's modification of Löwenthal's method. The potassium permanganate was standardized against ammonium oxalate, 1 gram of the latter being taken as equivalent to 0.4648 gram of tannin, as recently determined by Spiers.[8]

The tannin content was the same in both solutions; but the active solution before precipitation of the tannin took slightly more potassium permanganate than the control, due to the reducing action of soluble substances in the unboiled enzyme powder.

Amygdalin.

When placed in Raulin's solution containing 2 per cent of amygdalin without any other carbohydrate or nitrogenous compound present, the conidia of *Aspergillus terricola* produced considerable sterile growth in solution and a moderate light brown fruiting growth on the surface. In the same kind of solution containing nitrate in addition a thick hyphal mat was formed. This growth was converted into a powder by the usual treatment. Fifty cc. of a 1 per cent amygdalin solution were used to determine the activity of this powder. After five days' incubation the solution had a strong odor of benzaldehyde and gave a positive

[7] *Virginia Agricultural Experiment Station Report,* 1911–12, pp. 51–77.
[8] *Journ. Agricul. Sci.,* vi, pt. 1, p. 77, 1904.

test for hydrocyanic acid with Payer's reagent. Benedict's reagent showed the presence of 0.411 gram of glucose in 50 cc.

Five cc. of solution required 0.17 cc. of $\frac{N}{20}$ caustic soda to neutralize it with phenolphthalein as an indicator; the total acidity was therefore equal to 1.7 cc. of $\frac{N}{20}$ acid. Control solutions were tested in the same way and gave negative results, except for a very slight acidity equal to 0.2 cc. of $\frac{N}{20}$ acid for the 50 cc. of solution.

A powder from the growth on Raulin's peptone saccharose solution was also tested and formed the same quantity of $\frac{N}{20}$ acid as the enzyme powder in the preceding experiment. The control gave a negative result with Payer's reagent, while the active solution was strongly positive. The active solution was distilled to drive off the benzaldehyde and hydrocyanic acid; the residual solution formed a glucosazone with phenylhydrazine hydrochloride in fourteen minutes, while the control run in parallel gave no precipitate in forty-five minutes.

Ethyl alcohol.

One-tenth gram of enzyme powder prepared from a growth in a glucose solution was added to 25 cc. of water containing half a cc. of ethyl alcohol (99.75 per cent) and half a cc. of toluene. Boiled enzyme powder in a similar solution was used as a control. After four days' incubation the check required 0.4 cc. of $\frac{N}{20}$ caustic soda solution and the active solution 1 cc. to neutralize the whole 25 cc. The raw enzyme powder contained the same quantity of acid as the boiled one. This gain of 0.6 cc. of $\frac{N}{20}$ acid was due to the presence of an alcoholoxydase. The powder from Czapek's solution, when tested in the same way, gave no increase in acidity.

Olive oil.

An olive oil medium was prepared by shaking in a test-tube 3 cc. of olive oil and 5 cc. of melted agar until the agar had about set, and then pouring this emulsion into plain Raulin's solution. The oil globules were thus kept separated for a time and so a greater oil surface was exposed and the air supply not cut off from the solution, as it is when covered with a layer of oil. This emul-

sion gradually broke down and large oil globules formed, but the mold grew around them so that they did not form a film covering the surface of the solution. In three weeks an abundant umbrinous growth had formed around the edge of the solution and was adhering to the flask. The hyphae formed a dense mat around the drops liberated by the agar. An enzyme powder was prepared from this growth and the activity of 0.1 gram of it was tested in a suspension consisting of 24 cc. of water, 0.5 cc. of olive oil, and 0.5 cc. of toluene. After five days the oil suspensions were filtered; 10 cc. were pipetted into a porcelain dish, two drops of phenolphthalein added, and the acidity was determined with $\frac{N}{20}$ caustic soda. The acidity of the entire active solution was equal to 0.43 cc. of $\frac{N}{20}$ acid, while the control required 0.24 cc., a gain of 0.19 cc.

Enzyme powder prepared from the growth on Raulin's peptone saccharose solution was added to an olive oil suspension like the one described. After five days' incubation the active and control solutions were filtered and their acidity was determined in the usual way. The control olive oil suspension was neutral, while the active one required 0.55 cc. of $\frac{N}{20}$ caustic soda to neutralize it. Twenty-five cc. of distilled water containing 0.1 gram of the raw enzyme powder required 0.2 cc. of $\frac{N}{20}$ alkali. The total acidity due to the hydrolysis of oil was therefore equal to 0.35 cc. of $\frac{N}{20}$ acid.

The fungous felt remained in the olive oil medium so long that some of the enzyme may have passed into the suspension as extracellular enzyme. This loss would account for the hydrolysis by the powder from this source being lower than that from the peptone saccharose medium. The lipolytic activity of both powders may have been reduced by the *Acetonedauerhefe* method of preparation, as Currie[9] found to be the case in his work with the lipase of *Penicillium roqueforti.*

Ethyl butyrate.

The enzyme powder from the growth on olive oil was added to 25 cc. of water containing half a cc. of ethyl butyrate, and half a

[9] James N. Currie: Flavor of Roquefort Cheese, *Journ. Agricul. Research, U. S. Department of Agriculture*, ii, pp. 1–14, 1914.

cc. of toluene. The solution was filtered after five days' incubation and 10 cc. were titrated with $\frac{N}{20}$ caustic soda, with phenolphthalein as an indicator. The entire control solution required 1.06 cc. and the active solution 6.8 cc. to neutralize it. The powder from the fungous mat formed on Raulin's peptone saccharose solution was tested in ethyl butyrate solutions of the above concentration, and after five days' incubation required 4.2 cc. of $\frac{N}{20}$ alkali to neutralize the control and 8.5 cc. for the active solution. As the raw powder took 0.2 cc., this was a gain of 4.1 cc. of $\frac{N}{20}$ acid due to hydrolysis by the enzyme.

Proteins.

The proteolytic activity of the powder prepared from the growth on Raulin's peptone saccharose solution was determined by adding it to sterile water containing cubes of coagulated egg-albumen (1 part egg-albumen to 2 parts water), Dunham's solution, gelatin and milk.

The cubes of egg-albumen were cut so that they measured 1 cm. on an edge. A white powdery precipitate gradually settled from the cubes in the active solution and after five days the solution was opalescent, the cubes were yellow, and their volume was about half the original size, the actual measurements being 0.75 by 0.85 by 0.9 cm. = 0.574 cc. Ammonia was present at this time, but no tryptophane, which was found quite strong, however, in a test made eleven days later. Dunham's solution also gave only ammonia after five days and tryptophane after sixteen days. No tests were made between these times, but tryptophane was formed much before this time, as it was usually quite strong. Gelatin was partly liquefied in five days and gave the same results as the preceding substances in the ammonia and tryptophane tests. Milk was coagulated in one day, digestion immediately setting in, so that at the end of five days only a very small quantity of curd remained. The qualitative tests gave results like those above.

Amines and amides.

Aspartic acid and urea were the substances used for these tests *Aspergillus terricola* produced a luxuriant growth covering the entire surface of the liquid when either of these substances was supplied as a source of nitrogen in Raulin's solution.

Powders from the growth on Raulin's peptone saccharose solution and on Czapek's solution were tested for the presence of amidase by adding them to 0.3 gram of urea or 0.665 gram of aspartic acid in 50 cc. of water. The urea was added as 10 per cent solution and the aspartic acid weighed and added directly. The controls were prepared in the usual way. The ammonia was determined after three days by the Folin aeration method, the product being nesslerized and read in the colorimeter. All solutions were run in duplicate. The results, expressed in parts per million, were as follows:

Urea.

SOURCE OF ENZYME POWDER	ACTIVE SOLUTION	CONTROL SOLUTION	INCREASE
Raulin's medium	2.84	2.1	0.74
Czapek's medium	73.3	1.1	72.2

Aspartic acid.

SOURCE OF ENZYME POWDER	ACTIVE SOLUTION	CONTROL SOLUTION	INCREASE
Raulin's medium	4.28	3.0	1.28
Czapek's medium	3.60	3.48	0.12

Nitrogen-fixing power.

The nitrogen-fixing power of this fungus was tested in nutrient solutions containing a carbohydrate or nitrogenous substance either alone or together. Glucose, mannite, peptone and ammonium sulphate were used in quantities varying from 0.025 to 5.0 per cent. After a month's incubation the solutions showed no gain in total nitrogen; in most cases there was a slight loss.

SUMMARY.

Aspergillus terricola produced inulase, diastase, invertase, maltase, alcoholoxydase, emulsin, lipase, protease, and amidase when grown in a medium without these substances.

It is evident from the number of enzymes formed that filamentous fungi as well as bacteria may be concerned in the production of the various organic decomposition products which have been isolated from the soil.

Many of the products of enzymic action are excellent sources of carbon and nitrogen for bacteria.

The ammonia produced by the fungus from protein material may, in the form of a salt, be directly assimilated by some plants.

The living organism hydrolyzes a small amount of cellulose in cellulose agar, but the presence of cellulase could not be demonstrated in an enzyme powder.

Neither lactase nor zymase were present in a powder from the growth in lactose and glucose solutions respectively.

Tannase was produced by the fungus when grown in a tannin solution.

The fungus showed no nitrogen-fixing power in nutrient solutions containing either a carbohydrate (dextrose or mannite) or a nitrogenous substance (peptone or ammonium sulphate) or both together.

STUDIES IN ENDOGENOUS URIC ACID METABOLISM.

By G. W. RAIZISS, H. DUBIN and A. I. RINGER.
(From the Department of Dermatological Research, Philadelphia Polyclinic and College for Graduates in Medicine, Philadelphia, Pa., Dr. Jay F. Schamberg, Director.)

(Received for publication, October 2, 1914.)

It is now generally accepted that the uric acid output of a human individual on an unrestricted diet is dependent upon the amount of purin material ingested, and upon the degree of cellular nuclein catabolism. The uric acid that is derived from the ingested nucleins is known as the exogenous fraction, and that which is derived from the body's own nucleins is known as the endogenous fraction.

Burian and Schur[1] were the first to draw this distinction in uric acid metabolism, and their view was soon accepted by most investigators. They performed two experiments on two normal individuals; the first experiment consisted of four periods of four days each, the second experiment of two periods of four days each.

In the first experiment it was found that on a diet of milk, eggs, cheese, bread and butter, the uric acid nitrogen elimination per day was 0.190 gm. On reducing the protein intake to the extent of about 55 per cent, there was found no diminution in the uric acid elimination.

On the basis of this, the authors concluded that the endogenous uric acid elimination is constant for any given individual, and not influenced by changes in diet, as long as it is purin-free.

In the second experiment they found an elimination of only 0.131 gm. of endogenous uric acid nitrogen, which led them to conclude that the endogenous uric acid elimination is different for different individuals.

In support of the last view they compiled a table showing the uric acid nitrogen output of different individuals on meat-free diets observed by different investigators up to the year 1900. The individual differences fluctuate between 0.075 and 0.200 gm. per day.

Simultaneously with Burian and Schur, Siven[2] performed a series of experiments which led him to conclusions similar to those of Burian and Schur. For a period of seventeen days he lived on a diet consisting of 600

[1] Burian and Schur: *Arch. f. e. ges. Physiol.*, lxxx, p. 241, 1900.
[2] Siven: *Skand. Arch. f. Physiol.*, xi, p. 123, 1900.

gm. of potatoes, 120 gm. of butter, 400 gm. of apples, 170 gm. of sugar and 330 gm. of beer, containing in all 2.83 gm. of nitrogen. The average uric acid nitrogen elimination during this period was 0.144 gm. per day.

In the second period of four days the diet was the same with the addition of one egg per day. The average uric acid nitrogen output was 0.149 gm. per day.

In the third period the diet consisted of 500 gm. of eggs, 450 gm. of potatoes, 90 gm. of butter, 660 gm. of beer and 400 gm. of apples,—a daily nitrogen content of 12.56 gm. During the period of seven days, the average uric acid nitrogen elimination was 0.147 gm. per day.

In the last period of six days the diet consisted of 400 gm. of eggs, 600 gm. of milk, 125 gm. of bread, 180 gm. of cheese, 30 gm. of butter, 100 gm. of apple, 330 gm. of beer, making in all a daily nitrogen intake of 22.63 gm. The average uric acid nitrogen output was 0.159 gm. per day.

From these results the author concluded that on a meat-free diet the amount of uric acid eliminated is constant, irrespective of the amount of protein in the food, and that it is entirely endogenous.

For the past two years we have had an opportunity to study the metabolism of individuals afflicted with psoriasis. We consider these patients normal as far as uric acid metabolism is concerned, because the various stages of the disease did not seem to affect the uric acid excretion in any way. In the course of our studies we found that these patients improved remarkably when kept on a meat-free low protein diet. A number of patients were kept on such a diet for months, and since, according to Burian and Schur and Siven, this diet may be considered purin-free, it offered an excellent opportunity for the study of the endogenous uric acid metabolism.

The patients were kept in private rooms at the Polyclinic Hospital. The urine was collected in glass-stoppered bottles and preserved with toluene. The daily periods were closed at the same hour each morning. The food was prepared in the laboratory by a special nurse and weighed amounts were given to the patient. Samples of the food were analyzed for their nitrogen content.

The total nitrogen was determined by Kjeldahl; creatinine by Folin; uric acid by the Folin-Shaffer method.[3]

[3] The filtration of ammonium urate through filter paper consumes a great deal of time. The following scheme was found to be serviceable, time-saving, and productive of as good results as the filtration through filter paper method. An ordinary glass funnel of 60 mm. diameter is inserted into a suction flask with a perforated rubber stopper. The funnel

During the course of our investigation, the uric acid metabolism was studied in ten individuals, of whom eight were psoriatic and two were normal individuals who served as controls.

PATIENT XI. M. L. Male. The patient, 28 years of age, has been afflicted with psoriasis for 14 years. He entered the hospital on September 27, 1913, between which time and January 7, 1914, when the present experiment was inaugurated, his metabolism was studied and the influence of different diets on the course of his disease was noted. Between September 30 and November 3 he was kept on a high protein meat-containing diet. Between November 4 and January 6 he was on a meat-free vegetable diet. From January 7 to March 4 the patient was on a meat-free low protein vegetable diet. The articles of food consumed during that period were bread, butter, cream, bananas, grapes, corn starch, potatoes, beets, celery, apples, turnips and oranges. During the last three weeks the nitrogen intake was increased by the addition of milk.

PATIENT IX A. J. H. Female. This patient, 18 years old, has suffered from psoriasis since the age of 15. The metabolism studies with which we are here concerned were started on May 13 and continued up to June 25, 1913. During this entire period the patient was kept on a vegetable (meat-free) diet.

In January, 1914, the same patient (IX B) returned to the hospital with a widespread outbreak of the disease. She was immediately put on a very low protein and meat-free diet. The uric acid studies were started on January 12 and continued until March 30.

PATIENT III. B. L. Female. The patient, 18 years of age, has suffered from psoriasis for five years. She is thin and poorly developed. On admission to the hospital she suffered from an exceedingly severe and widespread eruption. The uric acid metabolism was studied from February 19 to March 3, during which time she was kept on a vegetable and fruit diet.

During this period, W., a normal woman, an employee of the hospital, was kept on exactly the same diet as Patient III, and the protein and uric acid metabolisms were studied.

Patients V, X, XII and XIV were afflicted with psoriasis and had been kept on a vegetable diet for some time previous to the period in which the uric acid metabolism was studied.

In Table I the averages per day for the different periods are given.

---

is half filled with absorbent cotton, moistened, and suction applied. The cotton is pressed down tightly and is then covered with a thin layer of asbestos, which, after filtration is completed, can be easily separated from the cotton. Filtration of ammonium urate proceeds very rapidly. A series of twelve can be finished within 30 minutes. The asbestos layer is removed from the cotton plug and placed in the original flask. The procedure is then continued according to the Folin-Shaffer method. The asbestos does not interfere with the potassium permanganate titrations.

TABLE I.

DATE	NITROGEN IN FOOD	NITROGEN IN URINE	NITROGEN IN FECES	TOTAL N EXCRETED	NITROGEN BALANCE	URIC ACID NITROGEN	CREATININE NITROGEN	BODY-WEIGHT
Patient XI. M. L. Male.								
1914	gm.	gm.	gm.	gm.	gm.	gm.	gm.	kgm.
Jan. 7–Jan. 12....	5.61	3.72	1.46	5.18	+0.43	0.106		64.80
Jan. 13–Jan. 19....	5.97	3.82	1.58	5.40	+0.57	0.092	0.441	
Jan. 20–Jan. 26....	6.12	3.93	1.58	5.51	+0.61	0.088	0.416	64.50
Jan. 27–Feb. 2....	6.19	3.75	1.72	5.47	+0.72	0.088	0.414	64.30
Feb. 3–Feb. 9....	5.50	3.78	1.62	5.40	+0.10	0.096	0.424	64.00
Feb. 10–Feb. 16....	8.92	5.94	1.14	7.08	+1.84	0.094	0.414	63.50
Feb. 17–Feb. 23....	8.72	6.99	0.69	7.68	+1.04	0.098	0.393	63.10
Feb. 24–Mar. 4....	8.63	5.17				0.100	0.399	62.60
Patient IX A. J. H. Female.								
1913								
May 13–May 20....	7.02	5.37	1.74	7.11	−0.09	0.129	0.349	53.96
May 21–May 29....	7.86	4.65	1.24	5.89	+1.97	0.129	0.358	54.53
May 30–June 5....	4.29	2.99	1.93	4.92	−0.63	0.145	0.343	54.60
June 6–June 12....	5.16	2.47	1.89	4.36	+0.80	0.101	0.328	54.54
June 13–June 15....							0.298	
June 16–June 19....							0.297	52.90
June 20–June 25....	13.45	8.47	2.06	10.53	+2.92	0.104	0.272	53.80
Patient IX B. J. H. Female.								
1914								
Jan. 12–Jan. 16....	5.60						0.371	54.80
Jan. 21–Jan. 26....	5.50	3.79	1.59	5.38	+0.12	0.093	0.353	54.80
Jan. 27–Feb. 2....	5.66	3.40	1.37	4.77	+0.89	0.092	0.332	54.50
Feb. 3–Feb. 9....	4.91	3.17	1.09	4.26	+0.65		0.326	54.50
Feb. 10–Feb. 16....	5.19	3.25	1.48	4.73	+0.46	0.107	0.324	54.60
Feb. 17–Feb. 23....	4.73	3.20	1.35	4.55	+0.18	0.107	0.299	54.10
Feb. 24–Mar. 2....	4.27	2.96	1.07	4.03	+0.24	0.093	0.289	54.00
Mar. 3–Mar. 9....	4.84	3.18	1.52	4.70	+0.14	0.100	0.299	54.10
Mar. 10–Mar. 16....	5.26	3.45	1.51	4.96	+0.30	0.101	0.283	54.50
Mar. 17–Mar. 23....	5.37	2.89	1.09	3.98	+1.39	0.095	0.266	54.80
Mar. 24–Mar. 30....	4.24	2.62	1.31	3.93	+0.31	0.075	0.271	55.40
Patient III. B. L. Female.								
1913								
Feb. 19–Feb. 25....	7.61	4.06	1.51	5.57	+2.04	0.109	0.183	39.65
Feb. 26–Mar. 3....	6.81	4.76	1.02	5.78	+1.03	0.117	0.179	

TABLE I—Continued.

DATE	NITROGEN IN FOOD	NITROGEN IN URINE	NITROGEN IN FECES	TOTAL N EXCRETED	NITROGEN BALANCE	URIC ACID NITROGEN	CREATININE NITROGEN	BODY-WEIGHT
Normal Subject. W. Female.								
1913	gm.	gm.	gm.	gm.	gm.	gm.	gm.	kgm.
Feb. 19–Feb. 25....	7.52	6.79	1.78	8.57	−1.05	0.091	0.329	60.20
Feb. 26–Mar. 3....	6.95	6.16	0.92	7.08	−0.13	0.094	0.322	60.00
Patient V. B. Male.								
Feb. 12–Feb. 18....	8.23	5.28	1.17	6.45	+1.78	0.137	0.435	68.30
Patient X. McG. Female.								
Nov. 11–Nov. 17....	4.13	2.62	0.96	3.58	+0.55	0.107		48.60
Patient XII. S. Female.								
Nov. 11–Nov. 17....	4.15	3.36	0.84	4.20	−0.05	0.096		49.40
Patient XIV. J. G. Female.								
Nov. 11–Nov. 17....	4.13	3.77	0.50	4.27	−0.14	0.099		49.80

On examining the figures of uric acid nitrogen, several facts strike us as of importance.

The absolute amount of uric acid nitrogen eliminated per day on a meat-free diet is in no case so high as was found by Burian and Schur, and in many cases by Folin.[4] Uric acid nitrogen output in Burian's case averaged 0.186 gram per day for a period of 9 days, and in Folin's experiments it was 0.18, 0.21 and 0.22 in the first three days of Table II; 0.18, 0.18, 0.18 in the first three days of Table III; 0.16, 0.15, 0.11, 0.11 in the first four days of Table IV, and 0.20, 0.18, 0.14, 0.16 in the first four days of Table V.

In some of Folin's cases the uric acid output declined even during the preliminary period of high protein feeding; in every case, however, there was a marked diminution in the uric acid elimination after the starch and cream diet was inaugurated. Folin assumes that the diminished protein intake was responsible for it.

[4] Folin: *Amer. Journ. of Physiol.*, xiii, p. 66, 1905.

As will be shown later, we agree with Folin that the protein metabolism is capable of influencing the uric acid output (this was also demonstrated recently in a very convincing way by Taylor and Rose[5]), but we do not agree with him in his statement that it was the *only* cause. We came to this conclusion because of the following facts:

Patient X of our series, a young woman weighing 45.2 kgm., entered the hospital on September 24, 1913. She was at once placed on a meat-free vegetable diet, which, according to Burian and Schur and others, constitutes a purin-free diet. Her diet contained an average of 9.35 gm. of nitrogen per day. The results were as follows:

TABLE II.

DATE	NITROGEN IN FOOD	NITROGEN IN URINE	URIC ACID NITROGEN	CREATININE NITROGEN
1913	*gm.*	*gm.*	*gm.*	*gm.*
Sept. 26	8.92	7.20	0.198	0.287
Sept. 27	9.47	7.67	0.122	0.266
Sept. 28	9.26	7.37	0.100	0.269
Sept. 29	9.43	7.03	0.190	0.246
Sept. 30	9.32	6.30	0.159	0.240
Oct. 1	9.51	6.50	0.142	0.259
Oct. 2	9.47	6.55	0.115	0.240
Oct. 3	9.27	5.78	0.124	0.240
Oct. 4	9.29	5.85	0.111	0.249
Oct. 5	9.33	6.13	0.091	0.259
Oct. 6	9.59	6.00	0.104	0.261

This patient was kept in the hospital after this for seven weeks. Of all the uric acid determinations that were made, none was above 0.112 gram of nitrogen per day.

We made similar observations on Patient IX B. She entered the hospital on January 10, 1914, and was immediately placed on a diet similar to the above, but containing less nitrogen (milk was excluded). The results were as follows:

[5] Taylor and Rose: this *Journal*, xviii, p. 519, 1914.

TABLE III.

DATE	NITROGEN IN FOOD	NITROGEN IN URINE	URIC ACID NITROGEN	CREATININE NITROGEN
1914	gm.	gm.	gm.	gm.
Jan. 12	6.03	6.26	0.196	0.358
Jan. 13	4.90	5.60	0.146	0.376
Jan. 14	6.00	5.28	0.136	0.381
Jan. 15	4.92	4.47	0.125	0.376
Jan. 16	6.04	3.66	0.109	0.366
Jan. 17	4.95	4.45		
Jan. 18	5.95	4.75		
Jan. 19	4.82	3.79		
Jan. 20	6.14	3.72		
Jan. 21	5.19	3.75	0.103	0.342
Jan. 22	5.97	3.75	0.103	0.342
Jan. 23	5.01	4.15	0.095	0.358
Jan. 24	5.79	4.15	0.095	0.358
Jan. 25	5.24	3.47	0.082	0.358
Jan. 26	5.81	3.47	0.082	0.358

For two months after this, the patient's uric acid output was studied daily and only on two occasions did it reach 0.121 and 0.120 gram of nitrogen per day.

These two experiments serve to illustrate the fact that the uric acid elimination immediately following the change from a mixed diet to a purin-free diet does not consist of endogenous uric acid only. From the fact that the uric acid output gradually declines until it reaches a level at which it stays indefinitely, we are inclined to conclude that the high figures during the first week or so are due to a "sweeping" out of accumulated purin bodies from the tissues and the blood.

This may also account for the marked differences in the figures obtained for uric acid on a purin-free diet by different investigatois on various individuals. Some investigators did not allow the body sufficient time to rid itself of accumulated purins, thus causing an apparently high endogenous uric acid output.

There is another factor which may influence the uric acid output on a vegetable (purin-free) diet. Siven,[6] for a period of nineteen days, studied the hourly distribution of uric acid elimina-

[6] Siven: *loc. cit.*

tion. He did not find any relationship between the digestive work and the uric acid output, but he did find that during the resting hours of the night the hourly uric acid elimination was about 50 per cent of the amount eliminated per hour during the day.

Siven did not come to any definite conclusion as to whether it was the muscular work during the day that was responsible for the higher uric acid elimination or whether some other factor was operative.

We performed one experiment which we believe throws some light on this question and adds additional evidence to the theory that muscular exertion *is* capable of causing an increase in the uric acid output.

The subject of this experiment was Mr. R. L. Knowles, an assistant chemist in our laboratory. He is a well-developed young man, 20 years of age; weight 124 pounds. On February 10, 1914, he was placed on a low protein vegetable diet and continued it for two weeks. Throughout this time the subject led a very regular life, assisting with the chemical work in the laboratory. On February 18 and 19 more violent exercises were taken, dancing and practice on the pipe organ, causing the subject to perspire profusely. As is seen from the table, there is an unmistakable rise in the uric acid output during these two days, and also on the day following.

In studying the endogenous uric acid metabolism of an individual, this factor seems worthy of attention, and may also be responsible for individual differences in the uric acid elimination as observed by different investigators.

These assumptions find further justification in the fact that all the individuals of our series, of different weight and stature and different muscular development, eliminated practically the same amount of uric acid when kept on the same conditions of diet and rest. Patient IX, whose initial weight was 64.8 kgm., was found to eliminate practically the same amount of uric acid as Patient III, whose weight was only 39 kgm. With but two exceptions (the first three periods of Patients IX A and V, see Table I) the uric acid output for the various individuals was remarkably constant. For the first exception we have been able to find no explanation; the latter, however, may be due to the fact that the patient was allowed to attend to his business for several hours a day.

TABLE IV.

DATE	NITROGEN IN FOOD	NITROGEN IN URINE	URIC ACID NITROGEN	CREATININE NITROGEN	REMARKS
			Period I.		
1914	gm.	gm.	gm.	gm.	
Feb. 10	5.38	10.6	0.160	0.502	
Feb. 11	6.17	Some	urine lost.		
Feb. 12	5.13	6.04	0.133	0.502	
Feb. 13	6.18	5.53	0.131	0.479	
Feb. 14	5.10	4.60	0.116	0.418	
Feb. 15	6.18	4.87	0.129	0.485	
Feb. 16	5.15	4.51	0.128	0.457	
Feb. 17	5.67	5.43	0.130	0.467	
Feb. 18	4.50	4.62	0.140	0.457	Physical exercise.
Feb. 19	5.58	3.83	0.158	0.448	
Feb. 20	4.46	5.56	0.142	0.442	
Feb. 21	5.54	4.53	0.123	0.442	
Feb. 22	4.39	4.10	0.122	0.468	
Feb. 23	5.32	4.16	0.122	0.479	
Average per day on low protein diet			0.133 (126)	0.467	
			Period II.		
1914					
Feb. 24	19.86	8.26	0.154	0.442	
Feb. 25	20.60	11.56	0.141	0.453	
Feb. 26	19.88	12.13	0.134	0.474	
Feb. 27	19.87	13.70	0.146	0.468	
Feb. 28	20.36	14.55	0.147	0.468	
Mar. 1	19.83	14.82	0.155	0.457	
Mar. 2	20.34	15.10	0.161	0.452	
Mar. 3	20.29	14.50	0.167	0.462	
Mar. 4	19.52	13.50	0.138	0.467	
Mar. 5	19.02	13.70	0.158	0.479	
Average per day on high protein diet			0.150	0.462	

On the subject of the last experiment we also studied the influence of a high protein intake on the uric acid output. During the first period of fourteen days, in which the subject lived on a low protein diet, the average uric acid nitrogen output per day

was 0.133 gram. If we exclude the days when the uric acid was higher due to work and the first day of the period, which was clearly due to a sweeping out of accumulated purins (see page 479), then the average uric acid nitrogen per day is reduced to 0.126 gram per day.

In the second period of ten days the nitrogen intake was increased to about 20 grams per day by adding milk, eggs and cheese to the diet. The average uric acid nitrogen output rose to 0.150 gram. The results of this experiment corroborate the findings of Folin and of Taylor and Rose.

In the foregoing it was shown that the uric acid elimination in human individuals kept on a vegetable (meat-free) diet, may be influenced by the diet immediately preceding the experiment, that work and high protein intake may increase it considerably, and that the uric acid elimination of different individuals is really not so variable nor so high as was found by previous investigators. Having succeeded in finding a condition in which the uric acid output remains uniform for a long period of time, we then studied the influence of a diet, as free from nitrogen as can conveniently be administered, upon the uric acid output.

An experiment was performed on Patient XI after he had lived on a meat-free diet for a period of two months, during which time the uric acid output was carefully studied. As is seen from Table I, the average uric acid output of the patient per day fluctuated between 0.088 and 0.106 gm. In Table V the details of the experiment are given. For the sake of convenience, we shall divide the experiment into a foreperiod (February 24 to March 6), an experimental period (March 7 to 11), and an afterperiod (March 12 to 16). The daily output of uric acid nitrogen during the foreperiod fluctuated between 0.086 and 0.115 gm. On March 7 was commenced the nitrogen-free diet which consisted of sago starch and cream, and which was continued for five days. On the first day of the experimental period, the uric acid nitrogen output was 0.088 gm., which is within the lower limits of normal fluctuation. On the second day, however, it decreased to 0.047, at which low level it remained with but slight fluctuation for the remainder of the period. This low elimination of uric acid nitrogen is very much below the level of normal fluctuation.

On March 12 the vegetable diet was resumed, but the total nitrogen intake was less than in the foreperiod, because milk was excluded. On the first day of the afterperiod the uric acid output was 0.056 gm., still below the ordinary. On the second day it reached its normal level and remained there thereafter.

TABLE V.

DATE	NITROGEN IN FOOD	NITROGEN IN URINE	URIC ACID NITROGEN	CREATININE NITROGEN	REMARKS
1914					
Feb. 24	8.16	5.42	0.108	0.400	
Feb. 25	9.07	6.27	0.087	0.400	
Feb. 26	8.47	5.30	0.103	0.415	
Feb. 27	9.03	5.25	0.086	0.391	
Feb. 28	8.34	5.00	0.099	0.394	
Mar. 1	9.01	6.43	0.100	0.406	Foreperiod.
Mar. 2	8.34	4.35	0.107	0.382	
Mar. 3	8.87	4.23	0.115	0.410	
Mar. 4	8.42	4.32	0.097	0.398	
Mar. 5	5.33	5.09	0.113	0.406	
Mar. 6	4.69	3.73	0.110	0.394	
Mar. 7	0.58	3.63	0.088	0.386	
Mar. 8	0.60	3.49	0.047	0.427	Experimental
Mar. 9	0.60	3.78	0.042	0.437	period.
Mar. 10	0.50	3.44	0.044	0.426	Nitrogen-free diet.
Mar. 11	0.50	2.28	0.052	0.397	
Mar. 12	4.70	2.39	0.056	0.394	
Mar. 13	4.95	2.79	0.097	0.394	
Mar. 14	4.35	3.74	0.095	0.379	Afterperiod.
Mar. 15	5.09	3.82	0.090	0.397	
Mar. 16	4.46	3.13	0.103	0.372	

A similar experiment was performed on Patient IX A. For five weeks prior to the commencement of the nitrogen-free diet period, the patient was kept on a vegetable (meat-free) diet. During the foreperiod (June 6 to 12) the average uric acid nitrogen elimination per day was 0.101 gm. On June 13, 14 and 15 the diet contained between 0.26 and 0.87 gm. of nitrogen per day. The uric acid nitrogen output dropped to 0.037, 0.039 and 0.040 gm. for each of the experimental days. On June 16, 17, 18 and 19 the diet consisted of milk, eggs and zwieback, with a nitrogen content of 11 to 12 gm. per day. The uric acid N elimination remained low,—0.042, 0.041, 0.058 and 0.052,—during each of the four days. On June 20 the ordinary vegetable (meat-free) diet was resumed, and the uric acid nitrogen output rose to an average of 0.104 gm. per day.

These two experiments are very significant in that they unmistakably show that a diet consisting of cream and starch causes a very marked drop in what is believed to be the endogenous uric acid output.

This drop in the uric acid elimination cannot be attributed solely to the drop in the nitrogen intake, for in patient IX A the nitrogen intake for four days following the nitrogen-free diet was raised to a level of 12 grams, with but a slight increase in the uric acid output.

The following consideration seems to offer an explanation for the behavior of uric acid elimination.

TABLE VI.

DATE	NITROGEN IN FOOD	NITROGEN IN URINE	URIC ACID NITROGEN	CREATININE NITROGEN	REMARKS
1913					
June 6–June 12	5.16	2.47	0.101	0.328	Vegetable diet.
June 13	0.87	2.50	0.037	0.297	Nitrogen-free diet; cream and starch.
June 14	0.26	1.65	0.039	0.298	
June 15	0.32	1.67	0.040	0.298	
June 16	6.28	2.30	0.042	0.389	Diet of milk, eggs and zwieback.
June 17	12.80	6.89	0.041	0.324	
June 18	11.82	10.01	0.058	0.289	
June 19	11.73	8.85	0.052	0.287	
June 20–June 25	13.45	8.47	0.104	0.272	Vegetable diet.

It is very possible that the true endogenous uric acid elimination per day is in reality much less than has heretofore been found, and that the only purin-free diet, yielding endogenous uric acid, is that which is administered during the experimental periods and consists of starch and cream, which are known to be free from uric acid-producing purin bases, and that the reason why more uric acid is eliminated on a vegetable diet than on a protein-free diet is because vegetables contain considerable amounts of nucleins which may yield considerable amounts of uric acid.

SUMMARY AND CONCLUSIONS.

I. The uric acid elimination of ten individuals was studied. It was found that on a vegetable (meat-free) diet the uric acid output of the different individuals was considerably lower than was previously reported in the literature.

II. It was also found that different individuals, under the same conditions of diet and rest, eliminate practically the same amount of uric acid per day.

III. Work and high protein intake cause an increase in uric acid elimination.

IV. In changing the diet from a mixed to a vegetable and meat-free diet, at least a week must be allowed before the uric acid output will reach a constant level. The output during the first few days is higher, in all probability, because of an elimination of accumulated purin in the tissues of the body.

V. The factors mentioned in III and IV may have been responsible for so many conflicting results reported in the uric acid literature and for the belief that different individuals eliminate different quantities of endogenous uric acid.

VI. On placing two individuals on a practically nitrogen-free diet, which was at the same time strictly nuclein-free, after a vegetable diet period, the uric acid elimination dropped to about half of what it was on the vegetable diet (0.040 gram per day).

VII. On giving milk, eggs and zwieback to the extent of 12 grams of nitrogen per day to one of these patients, immediately following the nitrogen-free diet, the uric acid output rose but very slightly (maximum of 0.058 gram per day). But when the vegetable diet was resumed, the uric acid nitrogen elimination rose again to its former level of 0.104 gram per day.

VIII. These findings suggest the possibility that the true endogenous uric acid output is really much smaller than has been found heretofore, and that in order to obtain it, the subject must be kept for a long time on a *strictly* purin-free diet and in a condition of absolute rest.

These requirements, of course, make researches in endogenous uric acid metabolism more difficult from the subject's as well as the operator's point of view. We hope, however, to be able to continue our inquiry into the various phases of this problem.

THE EXCRETION OF CREATININE BY HUMAN INDIVIDUALS ON A PROLONGED CREATINE-FREE DIET.

By A. I. RINGER and G. W. RAIZISS.

(From the Department of Dermatological Research, Philadelphia Polyclinic and College for Graduates in Medicine, Philadelphia, Pa., Dr. Jay F. Schamberg, Director.)

(Received for publication, October 2, 1914.)

In 1905 Folin[1] published his findings on creatinine excretion in the urine of a number of human individuals. He found that on a meat-free diet the creatinine elimination from day to day was practically constant for the same individual, but was different for different individuals and was entirely independent of the protein metabolism. On the whole, the creatinine elimination was found to be proportional to the muscular development and weight of the individual.[2]

In this communication it is our object to present the results of our observations on the creatinine elimination of human individuals kept on a prolonged creatine- and creatinine-free diet. Our studies were on patients who were afflicted with psoriasis and chronic eczema, and on whom the therapeutic effect of a low protein meat-free diet was studied. The details of our methods have been described in the preceding communication and in the *Journal of Cutaneous Diseases.*

The point of interest in our observations lies in the fact that in all of our experiments, which lasted several months, *there was noticeable a gradual and steady decline in the creatinine output per day. A decline that unquestionably lies outside the physiological fluctuations. It is independent of any changes in body-weight, and is associated with a steady decline in the creatinine coefficient.*

[1] Folin: *Amer. Journ. of Physiol.*, xiii, p. 66, 1905.

[2] Benedict and Myers: *ibid.*, xviii, p. 393, 1907; Shaffer: *ibid.*, xxiii, p. 1, 1908; Tracy and Clark: this *Journal*, xix, p. 115, 1914.

PATIENT VIII. Female. Severe case of psoriasis.[3] Throughout her stay in the hospital she was kept on a low protein meat-free diet. In Table VII are given the averages per day for the four weekly periods.

TABLE VII.

Averages per day.

DATE	NITROGEN IN FOOD	NITROGEN IN URINE	NITROGEN IN FECES	TOTAL N EXCRETED	NITROGEN BALANCE	CREATININE NITROGEN	BODY-WEIGHT	CREATININE COEFFICIENT
1913	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*kgm.*	
May 7–May 13*....	7.08	3.61	0.91	4.52	+2.56	0.301	58.8	5.1
May 14–May 20.....	7.49	2.89	2.00	4.89	+2.60	0.271	58.5	4.6
May 21–May 29.....	7.80	3.16	1.35	4.51	+3.29	0.263	58.1	4.5
May 30–June 5.....	4.39	1.88	1.97	3.85	+0.54	0.250	58.4	4.3

* For two weeks before the creatinine study was started, the patient had been on a creatine-free diet.

During the first week this patient eliminated an average of 0.301 gm. of creatinine nitrogen, yielding a creatinine coefficient of 5.1. During the second and third weeks the average elimination per day was 0.271 and 0.263 gm., respectively. The coefficient went down to 4.6 and 4.5. In the last week the average creatinine figure was 0.250 gm., yielding a coefficient of 4.3.

The patient maintained her body-weight during these four weeks. The creatinine coefficient, however, was reduced from 5.1 to 4.3, a decline of 16 per cent.

The reader has no doubt noticed the very marked nitrogen retentions of this patient. The causes of this were fully discussed in the communication referred to above.[4] We shall repeat here that these patients are afflicted with a disease that is characterized by a very marked proliferation of the epithelial cells of the skin, which become cornified and exfoliate, entailing the loss of considerable quantities of nitrogen. During the four weeks of this study, the patient lost 45.2 grams of nitrogen in the form of scales, which, in this case, reduces the positive nitrogen balance very considerably. This condition, however, has been found to bear no appreciable relationship to creatinine metabolism.

PATIENT IX A. Female. The details of the study of this case were presented in the preceding communication (page 476). During the first

[3] Described in detail in the *Journ. of Cut. Dis.*, xxxi, p. 802, 1913.

[4] *Loc. cit.*

week she eliminated an average of 0.349 gm. of creatinine nitrogen per day. The creatinine coefficient was 6.5. It remained at this level for three weeks when it gradually began to decline. In the last week the elimination had declined to 0.272 gm. with a coefficient of 5.1, a decline of over 21 per cent.

The patient was then discharged from the hospital. After seven months she returned with a widespread eruption of the disease, and the studies of her metabolism were continued. In Table I of the preceding communication the details of these studies are given under the caption "Patient IX B."

The average creatinine nitrogen elimination per day at the beginning of that experiment was 0.371 gram, yielding a coefficient of 6.8, which is very close to that obtained in this patient at the beginning of the first studies, and corroborates Folin's findings with regard to the constancy of the creatinine elimination for a given individual under ordinary circumstances. With the continuation of the meat-free vegetable diet there is seen a gradual decline in the creatinine output, which, during the last weeks of the experiment, reaches the low figures of 0.266, 0.271 and 0.276 gram per day. The creatinine coefficient drops to 4.9 and 5.0, a decline of almost 22 per cent.

Patient XIII. Male. Very severe case of generalized squamous eczema. The patient also suffered from hypostatic edema of the legs and feet which disappeared partially during the night and returned during the day. He did not show any evidence of nephritis, however. The patient was admitted to the hospital on January 14, 1914, and was placed on a meat-free vegetable diet. The nitrogen intake was varied by the addition or subtraction of milk.

TABLE VIII.

Averages per day.

Date	Nitrogen in food	Nitrogen in urine	Nitrogen in feces	Total N excreted	Nitrogen balance	Creatinine nitrogen	Body-weight	Creatinine coefficient
1914	gm.	gm.	gm.	gm.	gm.	gm.	kgm.	
Jan. 17–Jan. 26....	10.40	6.23	1.54	7.77	+2.63	0.448	63.8	7.0
Jan. 27–Feb. 2....	5.62	4.76	1.51	6.27	−0.65	0.422	63.2	6.6
Feb. 3–Feb. 9....	6.24	5.58	1.36	6.94	−0.70	0.407	60.9	6.7
Feb. 10–Feb. 16....	7.50	5.31	1.70	7.01	+0.49	0.385	59.5	6.5
Feb. 17–Feb. 23....	4.74	3.76	1.45	5.21	−0.47	0.348	59.0	5.9
Feb. 24–Mar. 2....	7.46	3.97	1.26	5.23	+2.23	0.335	58.2	5.7
Mar. 3–Mar. 9....	8.68	5.73	1.44	7.17	+1.51	0.344	59.3	5.8
Mar. 10–Mar. 16....	8.43	5.75	1.60	7.35	+1.08	0.359	59.3	6.0

As will be seen from the table, the patient lost considerably in weight during the first four weeks of his stay in the hospital; this was not due to insufficient caloric supply in the food, but to the disappearance of the edema.

During the first week the patient eliminated, on an average, 0.448 gram of creatinine nitrogen per day. Similar to the cases presented above, there is a gradual decline in its elimination, until, during the last three weeks, it descends to the low average of 0.335, 0.344 and 0.359 gram. In this case the decline is about 23 per cent.

There is also a decline in the creatinine coefficient in this case, but it is not as reliable as in the preceding cases, because of the edematous condition of the patient, which acts as a disturbing factor.

PATIENT XI. Male. Afflicted with psoriasis. The metabolic studies are reported in the preceding communication (page 476). There, too, a decline in the creatinine elimination is noticeable, although to a lesser degree than in the other cases.

SUMMARY AND CONCLUSIONS.

Five experiments are reported on four different individuals who were kept on creatine- and creatinine-free diets for long periods of time. Under these conditions it was found that the creatinine elimination was reduced to the extent of 16 per cent in Patient VIII, 21 per cent in Patient IX A, 22 per cent in Patient IX B, 23 per cent in Patient XIII and 10 per cent in Patient XI.

These conclusions are based on what may be termed a statistical method of study. Since the values of the *average* figures given in the tables depend entirely upon the extent of the fluctuation of the daily creatinine output, we append a table of the daily creatinine eliminations of the individuals studied. It may be mentioned here that the creatinine was always determined by one of us (G. W. R.).

An interpretation of the above results is at present difficult. In view of the revived interest in studies of creatinine metabolism during the past year, we consider the publication of these observations of interest.

TABLE IX.

The daily amounts of creatinine nitrogen eliminated.

PATIENT VIII	AVERAGE	CREATININE COEFFICIENT
May 7–May 13, 0.314, 0.304, 0.314, 0.301, 0.292, 0.287, 0.292	0.301	5.1
May 14–May 20, 0.253, 0.289, 0.273, 0.289, 0.264, 0.255, 0.271	0.271	4.6
May 21–May 29, 0.276, 0.260, 0.269, 0.266, 0.262, 0.251, 0.253, 0.279, 0.253	0.263	4.5
May 30–June 5, 0.242, 0.264, 0.247, 0.237, 0.247, 0.258, 0.254	0.250	4.3
PATIENT IX A		
May 13–May 20, 0.366, 0.335, 0.366, 0.363, 0.363, 0.334, 0.324, 0.346	0.349	6.5
May 21–May 29, 0.354, 0.358, 0.358, 0.347, 0.354, 0.367, 0.359, 0.365, 0.358	0.358	6.6
May 30–June 5, 0.335, 0.334, 0.327, 0.358, 0.346, 0.342, 0.357	0.343	6.3
June 6–June 12, 0.334, 0.358, 0.301, 0.331, 0.321, 0.324	0.328	6.0
June 13–June 15, 0.297, 0.298, 0.298	0.298	
June 16–June 19, 0.289, 0.324, 0.289, 0.287	0.297	5.6
June 20–June 25,* 0.276, 0.276, 0.267, 0.267, 0.273, 0.273	0.272	5.1
PATIENT IX B		
Jan. 12–Jan. 16, 0.358, 0.376, 0.381, 0.376, 0.366	0.371	6.8
Jan. 21–Jan. 26,† 0.342, 0.342, 0.358, 0.358, 0.358, 0.358	0.353	6.4
Jan. 27–Feb. 2, 0.324, 0.324, 0.324, 0.335, 0.335, 0.342, 0.342	0.332	6.1
Feb. 3–Feb. 9, 0.358, 0.358, 0.317, 0.317, 0.317, 0.308, 0.308	0.326	6.0
Feb. 10–Feb. 16, 0.317, 0.317, 0.327, 0.324, 0.321, 0.321, 0.342	0.324	5.9
Feb. 21–Feb. 23, 0.301, 0.304, 0.292	0.299	5.5
Feb. 24–Mar. 2, 0.281, 0.292, 0.289, 0.297, 0.301, 0.281, 0.286	0.289	5.4
Mar. 3–Mar. 9, 0.297, 0.307, 0.304, 0.284, 0.292, 0.307	0.299	5.5
Mar. 10–Mar. 16, 0.307, 0.287, 0.259, 0.266, 0.284, 0.304, 0.276	0.283	5.4
Mar. 17–Mar. 23, 0.276, 0.257, 0.255, 0.255, 0.281, 0.274	0.266	4.9
Mar. 24–Mar. 30, 0.262, 0.262, 0.278, 0.281	0.271	4.9
Mar. 31–Apr. 8, 0.279, 0.264, 0.281, 0.263, 0.268, 0.284, 0.278, 0.292	0.276	5.0

* Between June 20 and 25 the urine was analyzed in periods of two days.
† Between January 21 and February 11 the urine was analyzed in periods of two or three days.

TABLE IX—Continued.

PATIENT XIII		AVERAGE	CREATININE COEFFICIENT
Jan. 17–Jan. 26,‡	0.438, 0.418, 0.482, 0.453, 0.459, 0.453, 0.453, 0.442, 0.442, 0.442	0.448	7.0
Jan. 27–Feb. 2,	0.442, 0.432, 0.432, 0.419, 0.419, 0.405, 0.405	0.422	6.6
Feb. 3–Feb. 9,	0.418, 0.418, 0.409, 0.437, 0.413, 0.391, 0.364	0.407	6.7
Feb. 10–Feb. 16,	0.396, 0.382, 0.375, 0.383, 0.365, 0.372, 0.423	0.385	6.5
Feb. 17–Feb. 23,	0.398, 0.366, 0.341, 0.329, 0.334, 0.352, 0.319	0.348	5.9
Feb. 24–Mar. 2,	0.333, 0.329, 0.334, 0.329, 0.326, 0.337, 0.357	0.335	5.7
Mar. 3–Mar. 9,	0.356, 0.369, 0.356, 0.295, 0.343, 0.343, 0.349	0.344	5.8
Mar. 10–Mar. 16,	0.369, 0.365, 0.334, 0.329, 0.386, 0.332, 0.398	0.359	6.0

‡ Between January 22 and February 4 the urine was analyzed in periods of two days.

THE EFFECT OF ACID ON PERMEABILITY.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology of Harvard University.*)

(Received for publication, October 3, 1914.)

The effect of alkali on permeability has been discussed in a recent paper.[1] The writer has made similar investigations on the effect of acid, the results of which are here presented. As in the previous investigations the method employed was to make determinations of the electrical resistance of living tissues of *Laminaria saccharina;* these afford an accurate measure of the permeability of the protoplasm.

A number of lots of tissue were prepared in such a manner as to make them as nearly alike as possible. These were placed in sea water containing various amounts of HCl. The acid was added to the sea water in the form of a solution of HCl having the same conductivity as sea water (about 0.119 M HCl); in this way the conductivity of the sea water was not altered by the addition of the acid.

The effects of the various concentrations of acid are shown in Table 1 and Chart 1. It will be observed that the acid causes a rise in resistance; the greatest change occurred in sea water containing 0.015 M HCl in which the resistance rose 19 per cent in the course of five minutes.

We may regard the permeability as equal to the conductivity, or, in this case (for convenience) as equal to the net conductance. This is the reciprocal of the net resistance,[2] which is here expressed as percentage of the net resistance of the tissue in sea water at the beginning of the experiment. We may therefore put the permeability at the start as $1 \div 100 = 0.01$; at the end of

[1] This *Journal:* xix, p. 335, 1914.

[2] The net resistance is the resistance minus the resistance of the apparatus (*i.e.*, the resistance of the tissue itself). The net conductance is the reciprocal of this.

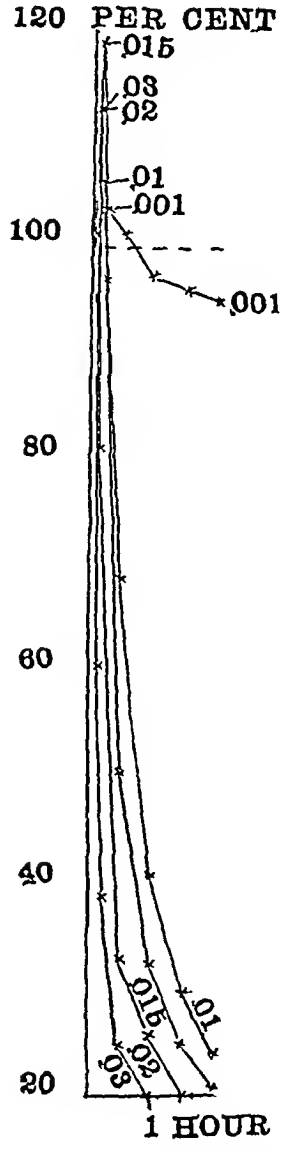


CHART 1. Curves of electrical resistance of *Laminaria saccharina* in sea water containing various amounts of HCl (unbroken lines) and of a control in sea water (dotted line). The ordinates represent net resistance expressed as percentage of the net resistance in sea water at the beginning of the experiment.

five minutes it was 1 ÷ 119 = 0.0084. The decrease in permeability is therefore 0.01 − 0.0084 = 0.0016, or 16 per cent.

It will also be seen that in higher concentrations (0.02 M and 0.03 M) the maximum was reached earlier than in 0.015 M, while in the lower concentrations (0.01 M and 0.001 M) it occurred later than in 0.015 M. It is evident that as the concentration increases the rise in resistance is more rapid and that the maximum point is passed more quickly. If the concentration be sufficiently increased, the period of increased resistance becomes shorter and shorter until it becomes difficult to detect it.

TABLE 1.

TIME IN MINUTES	ELECTRICAL RESISTANCE OF LAMINARIA SACCHARINA IN SEA WATER CONTAINING					
	0 HCl	0.001 M HCl	0.01 M HCl	0.015 M HCl	0.02 M HCl	0.03 M HCl
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0	100	100	100	100	100	100
1	100			107	113	113
5	100	102	104	119	103	100
10	100	104	106	90	80	60
30	100	101	69	71	33	25
60	100	97	41	33	26	20
90	100	96	30	25	20	
120	100	95	24	21		

All readings were taken at 18°C. The percentages were calculated on the basis of the net resistance in sea water at the beginning of the experiment.

The relation between concentration and changes in resistance is best shown in Chart 1a, in which the data given in Table 1 are plotted with the abscissae representing concentrations. From this it is evident that the effect of acid on permeability varies greatly not only with the concentration employed but also with the duration of the exposure. These relations could not be discovered without the employment of quantitative methods, in the absence of which conflicting results may easily be obtained.

In the previous experiments with alkali[3] it was found necessary to employ a solution containing NaCl + $CaCl_2$, since the addition of alkali to sea water causes a precipitate of magnesium hydrate. In order to compare the effect of acid with that of alkali a solution of acid having the same conductivity as the sea water was added to a solution containing 1793 cc. NaCl 0.52 M +

[3] *Loc. cit.*

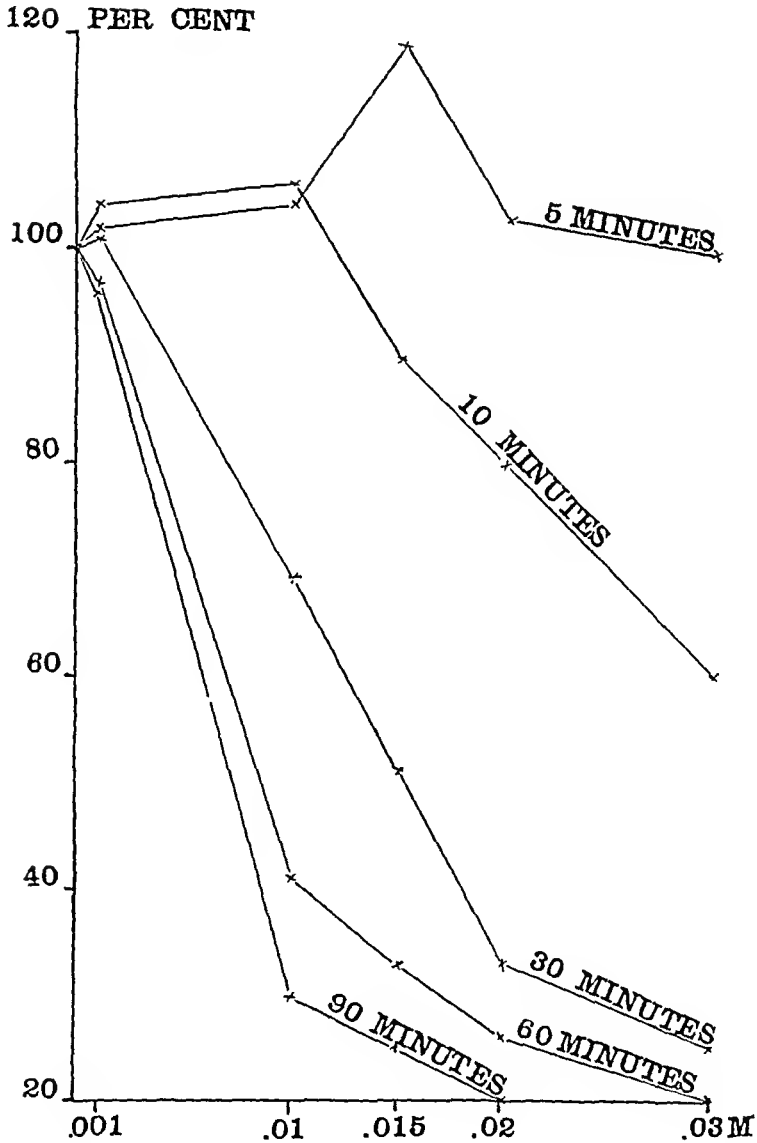


CHART 1a. Curves of electrical resistance of *Laminaria saccharina* in sea water containing various amounts of HCl. The ordinates represent net resistance expressed as percentage of the net resistance in sea water at the beginning of the experiment. The abscissae represent concentrations of HCl.

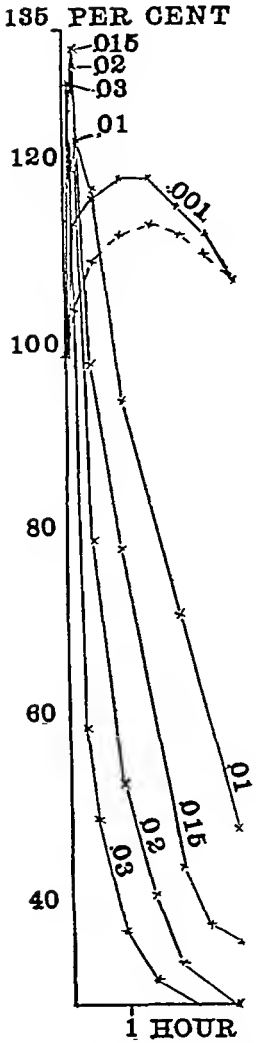


CHART 2. Curves of electrical resistance of *Laminaria saccharina* in (1793 cc. NaCl 0.52 M + 207 cc. $CaCl_2$ 0.279 M) containing various amounts of HCl. The ordinates represent net resistance expressed as percentage of the net resistance in sea water at the beginning of the experiment.

TABLE 2.

TIME IN MINUTES	ELECTRICAL RESISTANCE OF LAMINARIA SACCHARINA IN (1793 cc. NaCl 0.52 M + 207 cc. $CaCl_2$ 0.279 M) CONTAINING					
	0 HCl	0.001 M HCl	0.01 M HCl	0.015 M HCl	0.02 M HCl	0.03 M HCl
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0	100	100	100	100	100	100
5						129
10	105	114	120	133	131	
15	106		123			124
20	107					60
30	110	117	118	99	80	50
60	113	119	95	79	54	38
90	114	119			42	33
120	113	116	72	45	35	
150	111	113		39		
180	108	108	49	37	30	28

All readings were taken at 18°C. The percentages were calculated on the basis of the net resistance in sea water at the beginning of the experiment.

207 cc. $CaCl_2$ 0.279 M (this solution also had the conductivity of sea water). The results are shown in Table 2 and Chart 2.

It will be observed that the control shows a considerable rise in resistance; this is due to the comparatively large amount of

TABLE 3.

TIME IN MINUTES	ELECTRICAL RESISTANCE OF LAMINARIA SACCHARINA IN (1793 cc. NaCl 0.52 M + 207 cc. $CaCl_2$ 0.278 M) CONTAINING					
	0 HCl	0.001 M HCl	0.01 M HCl	0.015 M HCl	0.02 M HCl	0.03 M HCl
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0	100	100	100	100	100	100
2	100					125
10	100	109	114	127	125	
15	100		116			117
20	100					56
30	100	106	107	90	73	45
60	100	105	84	70	48	34
90	100	104			37	29
120	100	103	64	40	31	
150	100	102		35		
180	100	100	45	34	28	26

All readings were taken at 18°C. The percentages were calculated on the basis of the net resistance of the control.

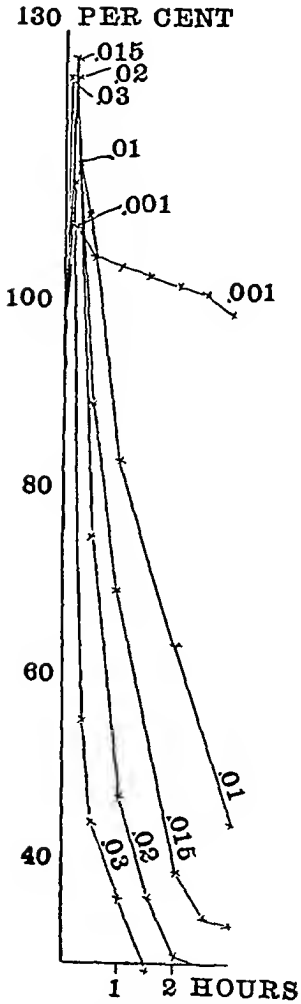


CHART 3. Curves of electrical resistance of *Laminaria saccharina* in (1793 cc. NaCl 0.52 M + 207 cc. $CaCl_2$ 0.279 M) containing various amounts of HCl. The ordinates represent net resistance expressed as percentage of the net resistance of the control.

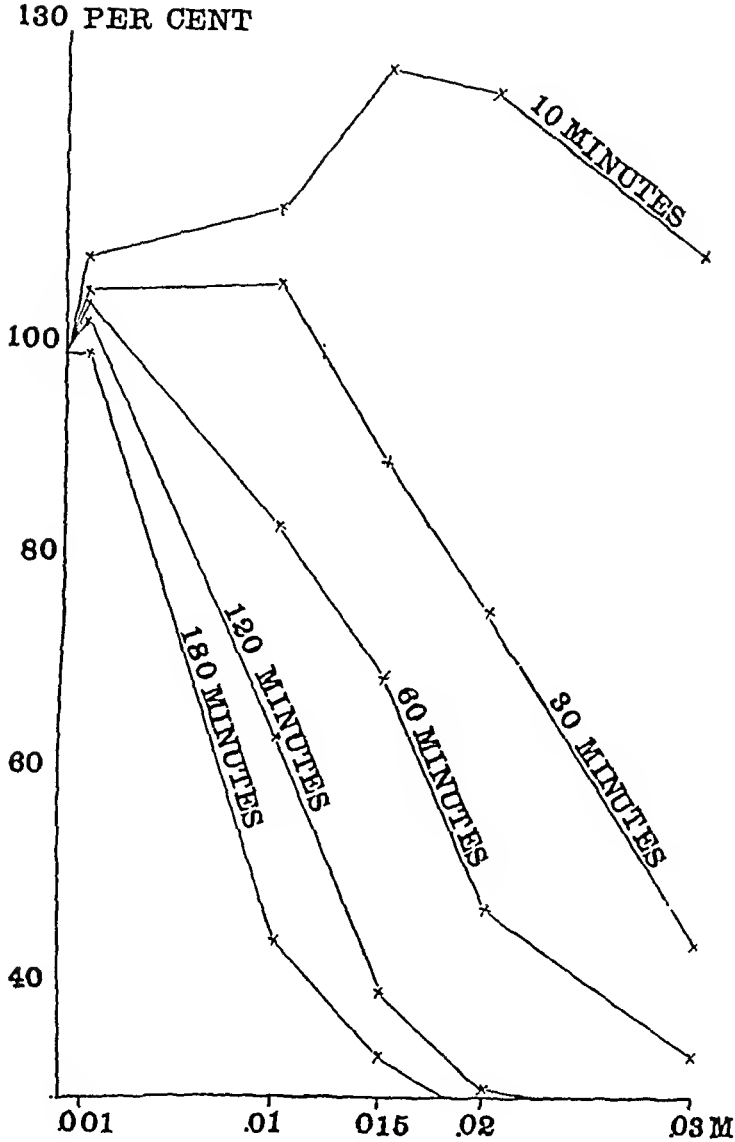


CHART 3a. Curves of electrical resistance of *Laminaria saccharina* in (1793 cc. NaCl 0.52 M + 207 cc. $CaCl_2$ 0.279 M) containing various amounts of HCl. The ordinates represent net resistance expressed as percentage of the net resistance of the control; the abscissae represent concentrations of HCl.

$CaCl_2$ present. For this reason the effect of the acid is more clearly seen if we express the resistance as percentage of the control. This is done in Table 3 and Chart 3. It will be noted that these results are in good agreement with those obtained by adding acid to sea water (as shown in Table 1 and Chart 1).

These relations are still more clearly shown when the results given in Table 3 are plotted in the manner shown in Chart 3a.

These results present a marked contrast with those obtained by the use of alkali;[4] the latter show no rise in resistance, but, on the contrary, a fairly rapid fall which continues until the death point is reached. This suggests certain analogies with the effects of acid and alkali on the physical properties of proteins. It would, however, be premature to discuss this more fully until further experiments can be made.

SUMMARY.

While alkali merely increases permeability, acid produces a rapid decrease of permeability followed at once by a rapid increase, which continues until the death point is reached.

[4] *Loc. cit.*

THE SOLUBILITY OF OXYGEN IN THE SERUM OF *LIMULUS POLYPHEMUS* L. AND IN SOLUTIONS OF PURE *LIMULUS* HAEMOCYANIN.

By CARL L. ALSBERG and WILLIAM MANSFIELD CLARK.

(From the Laboratory of the United States Bureau of Fisheries, Woods Hole, Mass.)

(Received for publication, October 13, 1914.)

To haemocyanin, the copper-containing protein found in the blood of many Crustacea and Mollusca, the rôle of oxygen carrier in respiration is very generally attributed.

A full discussion of this question is given by von Fürth.[1] Winterstein,[2] subsequently, pointed out that most of the recorded determinations of the quantity of oxygen that may be held in the blood of invertebrates are of little value because gasometric methods of analysis were not used. Jolyet and Regnard,[3] Griffiths,[4] Henze[5] and Winterstein[6] are the only investigators who employed such methods of analysis. All of these investigators studied either whole or defibrinated blood. Winterstein was quite unable to confirm the findings of Griffiths. Henze found the oxygen capacity of freshly drawn *Octopus* blood to be 3.09 – 3.70 volume per cent, while Winterstein found 4.2 – 5.0 per cent. Neither investigator states whether the blood is decolorized under diminished pressure. Frederic[7] states that it loses its color in twenty-four to forty-eight hours if the blood is merely out of contact with the air. If, however, the blood be warmed gently, under

---

[1] O. von Fürth: *Vergleichende chemische Physiologie der niederen Tiere*, Jena, 1903.

[2] H. Winterstein: Zur Kenntnis der Blutgase wirbelloser Seetiere, *Biochem. Zeitschr.*, xix, p. 392, 1909.

[3] Jolyet and Regnard: Recherches physiologiques sur la respiration des animaux aquatiques, *Arch. de physiol.*, Ser. 2, iv, p. 584, 1872.

[4] A. B. Griffiths: On the blood of the invertebrata, *Proc. Roy. Soc. of Edinburgh*, xviii, p. 288, 1890–1; also xix, p. 116, 1892.

[5] M. Henze: Zur Kenntnis des Haemocyanins, *Zeitschr. f. physiol. Chem.*, xxxiii, p. 370, 1901.

[6] H. Winterstein: *loc. cit.*

[7] L. Frederic: Recherches sur la physiologie du poulpe commun (*Octopus vulg.*), *Arch. de zool. expér.*, vii, p. 547, 1878.

diminished pressure, the blood at once becomes colorless. The oxygen compound is capable of forming again in the presence of oxygen, giving the blood the characteristic blue. Kobert[8] makes the same general statement concerning haemocyanin, without specifying the species of animal studied. All observers are agreed that within the living *Octopus* the venous blood is pale, the arterial blood blue. Winterstein found that under diminished pressure venous *Octopus* blood sets free little oxygen, while arterial blood contains very nearly as much as blood saturated with oxygen by shaking with air. For the oxygen capacity of the blood of the spiny lobster, *Palinurus vulgaris*, and of the spider-crab, *Maia squinado*, Winterstein obtained very much lower values than for *Octopus*. This he attributed to the fact that the blood of these animals contains less haemocyanin than that of *Octopus*.

Henze was the only investigator who examined solutions of pure haemocyanin. His results are reported in a second paper upon the haemocyanin of *Octopus*,[9] in which he found the oxygen capacity of pure haemocyanin sufficient to account for the oxygen capacity of the blood of *Octopus*.

The oxygen capacity of these bloods is very much greater than the solubility of oxygen in distilled water, as determined by Winkler,[10] or of sea water, as determined by Dittmar,[11] or of ox serum, as determined by Findlay and Creighton.[12] These values have been gathered in Table II.

In this paper are recorded results of experiments on the serum of the blood of *Limulus polyphemus* L., and on solutions of haemocyanin prepared from *Limulus* blood.

Serum, rather than blood, was used because of the great difficulty of preventing *Limulus* blood from coagulating. The solutions of serum and of haemocyanin for examination were prepared as described by Alsberg and Clark.[13] Preliminary experiments were made on the serum prepared from animals stored in a floating car in Woods Hole harbor for two months before transportation to the laboratory. For all other determinations serum from the blood of freshly captured animals was employed. The freshly drawn blood, after standing in the ice-box an hour or more, was

[8] R. Kobert: Ueber Haemocyanin nebst einigen Notizen über Hämerythrin, *Arch. f. d. ges. Physiol.*, xcviii, p. 411, 1903.

[9] M. Henze: *Zeitschr. f. physiol. Chemie*, xliii, pp. 290–9, 1904–5.

[10] L. W. Winkler: *Ber. der deutsch. chem. Gesellsch.*, xxii, p. 1764, 1889.

[11] William Dittmar: Report on the composition of ocean water, in *Report of the Scientific Results of the Voyage of H. M. S. Challenger, Physics and Chemistry*, i, pt. 1, p. 224.

[12] Alex. Findlay and H. Jermain Creighton: *Biochem. Journ.*, v, p. 294, 1910–11.

[13] C. L. Alsberg and E. D. Clark: The haemocyanin of *Limulus polyphemus*, this *Journal*, viii, p. 1, 1910.

filtered on paper and used at once. The haemocyanin was prepared from such serum by precipitation with the requisite amount of saturated ammonium sulphate solution. The precipitate after dissolving in water and reprecipitating with ammonium sulphate, as detailed in the earlier paper by Alsberg and Clark,[14] was dialyzed under toluene to free it from adherent ammonium sulphate. Such solutions show faint acidity. In one of the experiments the solution was carefully neutralized with sodium hydroxide. When the ammonium sulphate was nearly removed, sodium chloride was added to prevent the precipitation of haemocyanin which is not very soluble in water containing low concentration of electrolytes. The haemocyanin concentration of the final solution, as estimated from its nitrogen content, determined by the Kjeldahl method, was found to be 10 per cent.

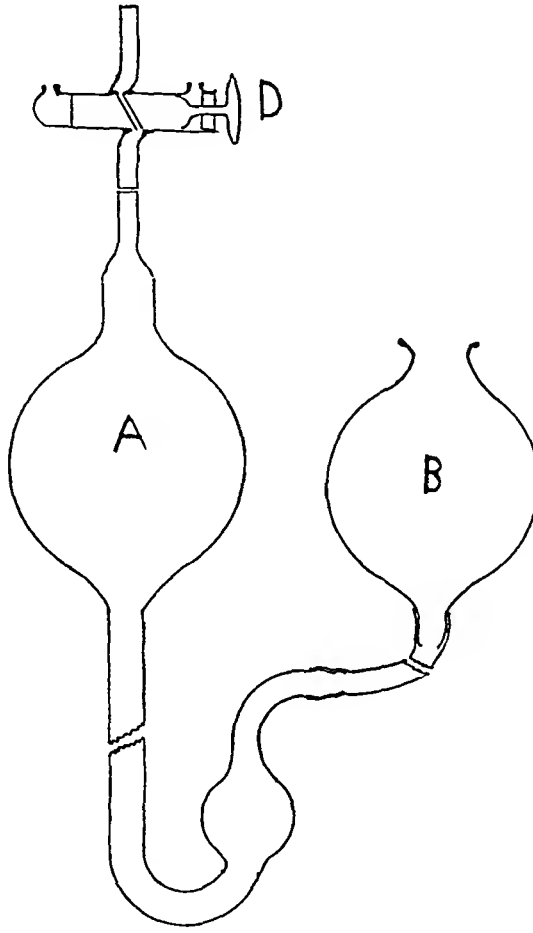
For the gasometric determinations the apparatus shown in the diagram was used. The bulb *A* was of sufficient size to hold 100 cc. of solution with ample space for frothing. It connected with a leveling bulb *B* through a tube about 80 cm. long. The bulb *A* was partially filled with mercury and the mercury in *A* and *B* brought to the same level by adjusting *B*. While the bulbs were kept in this position a burette containing the solution to be tested was attached to the upper opening of *A*. The stopcock of the burette was then opened and by the adjustment of *B* a very slight diminution of pressure, sufficient to allow a normal delivery from the burette, was maintained in *A*. When the proper quantity of solution had thus been introduced into *A*, connection was made to an Antropoff[15] pump. This connection, which is shown in the figure just below the mercury sealed stopcock *D*, was made with a piece of thick rubber tubing tied securely with waxed shoemaker's thread and completely surrounded by a mercury seal.

With the stopcock *D* open and the delivery tube of the pump clear of mercury and open to the air, *B* was raised slowly until the air in *A* had been expelled and the solution had risen to the top of the channel in the cock *D*. *D* was then closed and the pump evacuated to 0.02 mm. Hg, as measured by a McLeod gauge.

[14] C. L. Alsberg and E. D. Clark: *loc. cit.*

[15] Antropoff: *Chem.-Ztg.*, xxxiv, p. 979, 1910.

Meanwhile *B* had been lowered the barometric distance below *A*, and, in consequence, a considerable portion of the bulb *A* was left free for the foaming of the solution. When the pump had been evacuated, *D* was opened and the gas pumped over into a receiving vessel. The removal of gas was facilitated occasionally



by raising the liquid in the stopcock, whereby the gas which had collected was forced over into the pump. In the analysis of the gas a water-jacketed burette designed for the analysis of small quantities of gas produced by bacteria was used. Its reading limit was about 0.01 cc. The confining medium was mercury. Carbon dioxide was absorbed in 33 per cent KOH and oxygen in

Hempel's alkaline pyrogallol solution. In the recorded analyses all volumes of gas are for normal temperature, pressure, and dryness.

After preliminary experiments to ascertain the correct details for the procedure, a determination was made with 75 cc. of serum. Two more determinations were made of the gas content of the serum of recently captured animals. The results are given in Table I. The first determination, made with 75 cc., has been

TABLE I.

Analysis of gas from serum and haemocyanin of Limulus polyphemus L.

NO.			TEMPERATURE	BAROMETER	TOTAL VOLUME OF GAS	COMPOSITION OF GAS			VOLUME OF O_2 IN 1 cc.
						CO_2	O_2	N_2 by difference	
		per cent	*°C.*	*mm.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
1	Serum.....		27	762.5	2.82	1.73	0.43	0.66	0.0043
2	Serum.....		28	766.0	4.76	3.60	0.45	0.71	0.0045
3	Serum.....		28	764.0	5.27	4.04	0.49	0.74	0.0049
4	Neutralized haemocyanin	10	27	768.2	1.24	None	0.55	0.69	0.0055
5	Unneutralized haemocyanin	10	31	765.0	1.55	0.18	0.51	0.86	0.0051

recalculated to the basis of 100 cc. in order to make it directly comparable with the others.

It is quite evident that the determinations show that the amount of oxygen which can be removed from *Limulus* serum or from a solution of *Limulus* haemocyanin by diminishing the pressure is very small. The quantity is of the same order of magnitude as that obtainable from distilled water, from sea water, or from ox serum. For purposes of comparison these results have been grouped in Table II.

The low oxygen content of the *Limulus* haemocyanin solutions is contrary to expectations. It may be that a relatively stable oxygen compound of *Limulus* haemocyanin is formed, and that this does not dissociate readily at the low pressure attained with the mercury pump. This supposition is in harmony with the fact that the *Limulus* haemocyanin does not lose its color under diminished pressure, as one would expect did the oxyhaemocyanin

dissociate readily. If this suggestion is in accord with the facts, then in these determinations the oxygen-combining power of haemocyanin has not been measured at all. Though the oxygen-haemocyanin compound seems relatively stable under the mercury pump, it is not stable within the animal; for the blood as it flows out is pale and deepens in color as it absorbs oxygen from the air. At any rate, there seem to be no indications of the usual sort that *Limulus* haemocyanin can act exactly like haemoglobin as an oxygen carrier. From the reports of Henze it would seem that *Octopus* haemocyanin can act in this manner. It must,

TABLE II.

Comparison of the oxygen content of distilled water, sea water Limulus serum, Limulus haemocyanin and Octopus blood.

TEMPERATURE	BAROMETER	VOLUME OF O_2 SET FREE IN 1000 CC. OF				
		Distilled water	Sea water	Serum of *Limulus polyphemus* L.	10 per cent solution of haemocyanin of *Limulus*	Blood of *Octopus*
°C.	mm.					
0.0	760.0					14.40
0 0	760.0					11.10
15.0	760.0		5.830			
20.0	760.0		5.310			
24.8	760.0	5.762				
25.0	760.0		4.870			
27.0	762.5			4.500		
27.7	768.2				5.300	
30.0	760.0		4.500			

therefore, be of a fundamentally different character from *Limulus* haemocyanin. This is in agreement with the facts shown by Alsberg and Clark[16] that they do differ in chemical composition and properties.

In considering the respiration of marine animals it must be remembered that they live under conditions analogous to those of land animals at high altitudes as far as concerns their opportunities for obtaining oxygen. The sea animal is in contact with oxygen at low tension and under conditions in which diffusion is less rapid than in the atmosphere. Hence, the rate of absorption

[16] C. L. Alsberg and E. D. Clark: *loc. cit.*

of oxygen depends not only upon the avidity of the absorbent for this gas, but also upon the rate at which oxygen is furnished. Were the avidity of the blood for oxygen to exceed a certain degree, the blood would not carry more oxygen to the tissues. Similarly, a powerful pump would not deliver its full capacity if compelled to draw its supply of water through a capillary tube. In the same sense haemoglobin is unable to cope with the low oxygen tension of high altitudes.

It might be a distinct advantage for an animal living in such an environment as the sea to have a carrier which combines firmly with oxygen so that this gas may be absorbed even at low tensions. If, at the same time, the carrier is able to give up its oxygen within the organism, it will be far more efficient than an analysis of the blood gases would indicate. The method by which it gives up oxygen would have to be quite different from that by which haemoglobin supplies the tissues. The latter dissociates at the low oxygen tension existing in the tissues and allows its oxygen to diffuse gradually into the plasma. If oxyhaemocyanin holds its oxygen much more firmly than oxyhaemoglobin, it can not give up its oxygen in this way.

It may be that the haemocyanin acts as a carrier of oxygen in much the same way that colloidal salts of iron and manganese appear to act in the so called synthetic oxidases,[17] or, to cite simpler cases, as the inorganic salts of these metals do in well known chemical reactions. Such carriers do not generally part with their oxygen at low pressures, but they give it up readily when in contact with other substances which are themselves incapable of combining with molecular oxygen. If the copper of haemocyanin is to function in this way it should not be too firmly combined in the molecule, but must be easily dissociated. Indeed such appears to be the case. It is, furthermore, interesting to note that the blood of *Limulus* is distinctly alkaline, a condition which Wolff[18] and Dony-Henault[19] found to be favorable for the stimulation of the oxidizing action of the manganese in certain oxidases. If these suggestions have any basis in fact then the

[17] J. H. Kastle: The Oxidases, *Hyg. Lab. Bull.*, lix, p. 122, 1910.

[18] Wolff: *Compt. rend. Acad. d. sci.*, cxlvii, p. 745, 1908.

[19] Dony-Henault: *Acad. roy. de Belg., Bull. de la classe des sciences*, p. 105, 1908.

attempt to remove the oxygen from the blood of *Limulus* will not furnish a true index of the efficiency of the haemocyanin in respiration. The most promising method of estimating it correctly would be to determine the amount of oxygen that completely reduced blood will remove from a known volume of the gas, or to determine the extent of oxidation which blood saturated with oxygen can produce in certain substances oxidizable by catalytic action, but not through direct contact with molecular oxygen.

SUMMARY.

The serum of *Limulus polyphemus* L., under diminished pressure, sets free about as much oxygen as distilled water or sea water after saturation with oxygen under similar conditions of temperature and pressure. A 10 per cent solution of haemocyanin, which contains about three times as much haemocyanin as the serum,[20] sets free almost 20 per cent more oxygen than the serum. This increased amount is too slight to warrant considering oxyhaemocyanin as an oxygen carrier, assuming that the 10 per cent solution of haemocyanin contains about three times as much haemocyanin as the serum does. These observations are in striking contrast to those of Henze on *Octopus* blood, although the haemocyanin content of *Octopus* blood is only slightly less than that of the 10 per cent solution used in this work. It is suggested here that *Limulus* haemocyanin may be a more efficient carrier of oxygen than would appear from any gas analyses, since its combination with oxygen does not seem to dissociate appreciably, if at all, under low pressure. With the aid of the copper, oxygen may, perhaps, be transferred catalytically within the organism.

[20] C. L. Alsberg: Note on the Proteins of the Blood of *Limulus polyphemus* L., this *Journal*, xix, p. 77, 1914.

THE COAGULATION OF ALBUMEN BY PRESSURE.

By P. W. BRIDGMAN.

(*From the Jefferson Physical Laboratory of Harvard University, Cambridge.*)

(Received for publication, October 20, 1914.)

The purpose of this note is to state a fact of possible biological interest which I have discovered incidentally in the course of other work. If the white of an egg is subjected to hydrostatic pressure at room temperature, it becomes coagulated, presenting an appearance much like that of a hard boiled egg.

The albumen was enclosed in a nickel-steel case, and pressure transmitted to it by mercury. The high pressure apparatus was that which I have already described.[1] Pressure may be applied so slowly that the rise of temperature due to the compression is inappreciable. At room temperature (20°) the limits of pressure necessary to produce the coagulation were fairly well marked. A pressure of 5000 atmospheres (75,000 pounds per square inch) applied for thirty minutes produced a perceptible stiffening of the white, but little more; 6000 atmospheres for thirty minutes produced a coagulation in appearance like curdled milk; while 7000 for thirty minutes resulted in apparently complete coagulation, the white being capable of standing under its own weight. If the duration of the pressure of 5000 was increased to one hour, the coagulation was only slightly increased in amount. 3000 atmospheres applied for sixteen hours produced a barely perceptible thickening of the white. The effect of temperature, which is not large, seems to be such that the ease of coagulation increases at low temperatures, contrary to what one might expect. 6000 atmospheres applied at 0° for one hour produced a somewhat greater stiffening than would have been produced at 20°. Pressures considerably higher than 7000 did not alter the effect. The first time I tried the experiment was with a pressure of 12,000

[1] *Proc. Amer. Acad. of Arts and Sciences*, xlix, p. 627, 1914.

atmospheres for twenty minutes; the resulting product was indistinguishable in appearance from that produced by 7000. A pressure of 12,000 at 20° was high enough to compel the water to freeze to a modification of ice, ice VI, denser than water. It is interesting that the coagulated white had not apparently been affected by this freezing.

I have made no attempt to determine whether the nature of the coagulation produced by pressure is the same as that produced by heat. If one can judge by appearances, the two may be different. In the course of twenty-four hours there separates from the pressure-coagulated white a small quantity of some watery fluid, in which the coagulated part remains insoluble.

THE DISTRIBUTION OF ARSENIC IN A HUMAN BODY.

By FRANK P. UNDERHILL.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, Conn.)

(Received for publication, October 23, 1914.)

A legal inquiry into the cause of death of a young woman led to the discovery of the presence of considerable quantities of arsenic in the body and afforded an opportunity for a study of the distribution of the poison. Complete data of this character are not numerous and the significance of such studies both from the toxicological and medicolegal viewpoints is obvious.

The history of the case indicated chronic arsenic poisoning. The individual had been ill for a period of approximately six weeks, presenting symptoms typical of arsenic intoxication, such as loss of appetite, malaise, nausea, pain in abdomen, vomiting, diarrhoea, peripheral neuritis, motor paralysis of the extremities, and finally convulsions and death.

After being buried for a period of three months the body was exhumed. The box, casket, and clothing were only slightly damp and were not discolored. The body, that of a woman apparently 20 years of age, was in a remarkable state of preservation, the tissues being firm, normal in color and without odor other than that of formalin, the preservative employed. The pleural cavity contained fluid, but the abdominal cavity was dry. The only organ presenting an abnormal appearance was the liver which was pale yellow, resembling strongly the liver of "fatty degeneration." The bladder was empty. Upon opening the gastroenteric tract, which presented no perforations throughout, the only abnormality to be observed was a small hemorrhagic area in the antrum of the pylorus. The intestines contained a quantity of semi-liquid material. About 30 cc. of a black gritty mixture were obtained from the rectum. From the stomach were taken 200 grams of fluid containing little or no food residues, but

exhibiting the presence of a considerable quantity of arsenious oxide in a crystalline form, thus revealing the form of the ingested arsenic and indicating the administration of a dose of the toxic substance a short time previous to death.

Unless otherwise specified the entire organs were removed. They were immediately ground in a meat chopper, sampled, and preserved separately in alcohol. Arsenic was estimated according to the procedure of Johnson and Chittenden.[1]

The distribution of arsenic follows.

ORGAN	WEIGHT OF ORGAN	As_2O_3	As_2O_3 PER 100 GM. OF TISSUE
	gm.	*mgm.*	*mgm.*
Liver	1105	184.6	16.7
Stomach (tissue)	290	23.8	8.2
Intestines (tissue)	700	41.6	5.9
Kidneys	200	8.5	4.2
Spinal cord	25	0.9	3.6
Lungs	645	19.4	3.0
Brain	1190	17.4	1.4
Spleen	135	1.9	1.4
Pancreas	80	0.9	1.1
Muscles (thigh)	600	3.3	0.5
Heart	300	1.1	0.3
Uterus and appendages	85	Present	Present.
Bone (femur)	100	Present	Present.
Hair	15	Present	Present.

Portion of absorbed arsenic as As_2O_3 ..... 303.4 mgm.

Stomach contents	200	15.8
Intestinal contents	200	4.0
Rectal contents	30	7.7

Unabsorbed arsenic as As_2O_3 ..... 27.5 mgm.

The reported case presents some features of interest. In the first place, from its history and, in addition, from the presence of unchanged arsenious oxide in the stomach it may be accepted that the individual died from *chronic* arsenic poisoning. The relatively

[1] Johnson and Chittenden: *Amer. Chem. Journ.*, ii, p. 332, 1880-81.

large amount of the poison in the kidneys would also tend to substantiate this conclusion. According to Ludwig,[2] with arsenious oxide poisoning the greatest quantity of arsenic is found, as a rule, in the liver, a view confirmed by the present case. It is also of interest to note that when the amounts of arsenic are calculated to 100 grams of tissue the spinal cord is even richer in the poison than the lungs, spleen, muscle, brain, or pancreas, and further that the brain contained more arsenic than the pancreas, muscle, or spleen.[3] The even distribution of arsenic in the muscle is exemplified by the quantities found in the heart muscle and those of the thigh muscles, again indicating chronic poisoning. The same point, that of chronicity, is further emphasized by the presence of the poison in detectable amounts in the bones and hair.

The case under discussion presents one feature which is not in accord with previous published records; namely, the relatively high arsenic content of the spinal cord and brain. The view has been held for many years that when significant quantities of arsenic are found in the brain and cord a soluble form of arsenic was ingested, and the idea that chronic poisoning with insoluble arsenic compounds, such as arsenious oxide, may lead to a notable deposition in the nervous tissues has been denied.[4] In the present instance, however, *chronic* poisoning undoubtedly obtained, arsenious oxide being the agent employed, and yet noteworthy quantities of arsenic were found in the brain and cord. In view of these facts the conclusion may be drawn that at times, at least, *the repeated ingestion of arsenious oxide may lead to the deposition of significant quantities of arsenic in the brain and spinal cord.*

[2] Quoted from Chittenden: *ibid.*, v, p. 1, 1884.

[3] Cf. Ekeley: *Journ. Amer. Chem. Soc.*, xxxv, p. 483, 1913.

[4] Chittenden: *Amer. Chem. Journ.*, v, p. 1, 1884; Chittenden and Smith: *Studies from the Laboratory of Physiological Chemistry, Sheffield Scientific School*, 1884–85, p. 141.

ANTAGONISM BETWEEN ACIDS AND SALTS.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University.*)

(Received for publication, October 27, 1914.)

Loeb[1] has shown that salts are antagonized by acids and has pointed out that this has a special significance for the theory of permeability, since it indicates that the permeability of the plasma membrane (for water and substances soluble in water) depends on the presence of protein rather than of lipoid substances.

The writer has made investigations which show that similar (though less striking) antagonism occurs in plants. This affords evidence of the protein character of the plasma membrane in plants and is in harmony with the fact that (as the writer has shown) ions pass through the plasma membrane of plants,[2] which would not be expected if it were composed of lipoid.

The method employed in these investigations was to determine the electrical conductivity of *Laminaria saccharina;* this affords an accurate measure of the permeability of the protoplasm.

A solution of HCl having the same conductivity as sea water (about 0.119 M HCl) was prepared. Various amounts of this were added to a solution of NaCl 0.52 M (which had the same con-

[1] Loeb, in 1899, in an article in *Pflügers Archiv* (lxxv, p. 308), showed that acid antagonizes the effect of NaCl on the swelling of muscle and made the suggestion that the antagonism depends on the action of these substances on the proteins of the tissue. The researches of Pauli and his pupils, as well as those of Prokter, have since shown that this is the case (Cf. Loeb: *Biochem. Zeitschr.*, xlvii, p. 147, 1912). In his articles in the *Biochemische Zeitschrift* in 1911 (xxxiii, p. 489) and in 1912 (xxxix, p. 167) Loeb demonstrated this antagonism by experiments in which the length of life of *Fundulus* served as a criterion. In a subsequent article (*Biochem. Zeitschr.*, xlvii, p. 147, 1912) he explained this antagonism as due to a direct effect on permeability.

[2] *Science*, N. S., xxxv, p. 112, 1912; xxxvi, p. 350, 1912; *Plant World*, xvi, p. 129, 1913.

ductivity as sea water). Several lots of tissue were prepared with a view to making them as much alike as possible. One lot of tissue was placed in each of the mixtures of NaCl + HCl; tissue was also placed in pure NaCl and pure HCl.

The results are shown in Table 1 and Chart 1. It will be seen that in pure NaCl and in pure HCl the resistance fell rapidly, indicating injury; while in a mixture in which the dissolved molecules are 99.09 per cent NaCl and 0.91 per cent HCl the resistance fell less rapidly, indicating that this mixture was less injurious than either of the pure solutions. In other words, the salt and

TABLE 1.

Composition of the solution in molecular proportions	Electrical resistance of Laminaria saccharina					
	At start	After 1 min.	After 10 min.	After 30 min.	After 60 min.	After 120 min.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
NaCl	100	93	77	70	60	52
NaCl 99.82, HCl 0.18	100	96	82	78	72	63
NaCl 99.54, HCl 0.46	100	97	85	81	66	57
NaCl 99.09, HCl 0.91	100	98	94	83	50	38
HCl	100	124	35	25	20	20

All readings were taken at 18°C.

The percentages were calculated on the basis of the net resistance in sea water at the beginning of the experiment.

the acid have an antagonistic action. This antagonism may be expressed quantitatively (as explained in a previous article[3]) in the following manner: The ends of the antagonism curve are connected by a straight line[4] and an ordinate is erected at the point on the curve which is to be measured. For example, the ends of the 30 minute curve in Chart 1 are connected by the

[3] *Botanical Gazette*, lviii, p. 178, 1914.

[4] This should in many cases be a curved line, provided the pure solutions are not equally toxic. But in the present case the curvature would be small, and at the maximum point of the curve very small indeed. This line expresses the additive effect (*Botanical Gazette, loc. cit.*); *i.e.*, the effect which would be produced if there were no antagonism, but if each component of the solution acted independently.

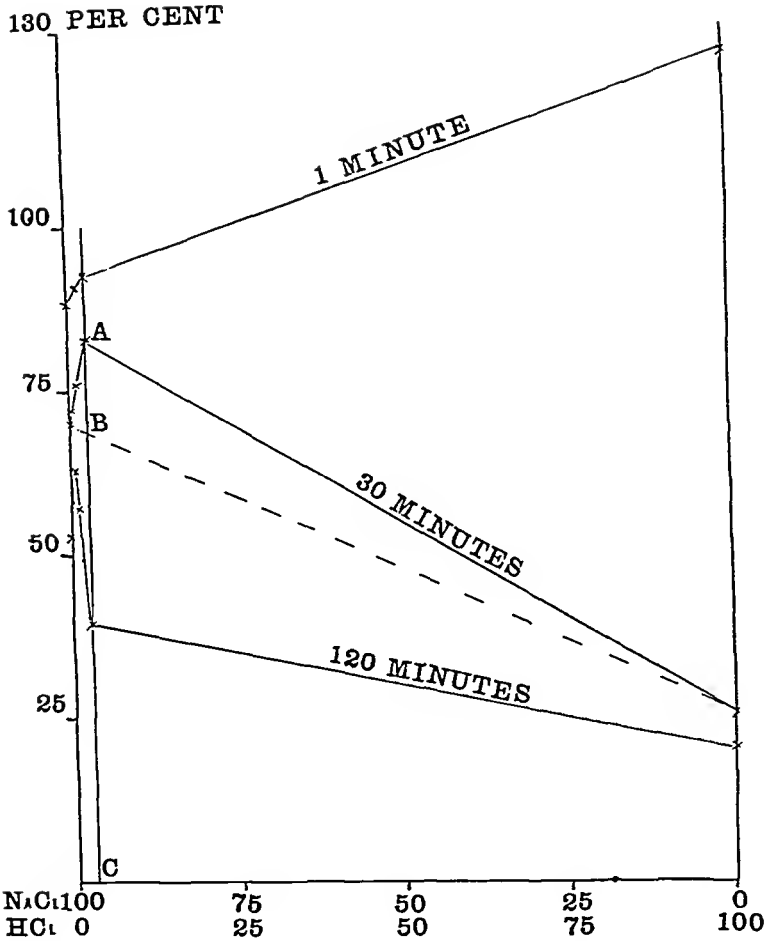


CHART 1. Antagonism curve of *Laminaria saccharina* in NaCl 0.52 M, in HCl 0.119 M, and in mixtures of these. The ordinates represent electrical net resistance (expressed as percentage of the normal net resistance); the abscissae represent the molecular proportions in the mixtures. Thus NaCl 50, HCl 50 means a mixture of NaCl 0.52 M and HCl 0.119 M in such proportions that 50 per cent of the dissolved molecules is NaCl and 50 per cent is HCl.

dotted line. The antagonism at the point *A* (representing a mixture in which the dissolved molecules are 99.09 per cent NaCl and 0.91 per cent HCl) is expressed as AB ÷ BC = 0.208. In the same way the antagonism at the maximum point on the 120 minute curve would be 0.19. The shift of the maximum toward the left (*i.e.*, from NaCl 99.09 + HCl 0.91 in the 30 minute curve to NaCl 99.82 + HCl 0.18 in the 120 minute curve) is not unusual in antagonism curves.

The rise in resistance at the end of 1 minute in pure HCl agrees with the results described in a previous paper.[5]

A reading taken at the end of 18 hours showed that the tissue was dead in all the solutions. The plants can be kept alive much longer than this in mixtures of NaCl + $CaCl_2$. It is also noteworthy that the degree of antagonism (as measured by the method previously described[6]) is greater in NaCl + $CaCl_2$ than in NaCl + HCl.

SUMMARY.

Acid can antagonize the action of NaCl.

The degree of antagonism is not as great as between NaCl and $CaCl_2$.

Life cannot be maintained as long in the most favorable mixtures of NaCl + HCl as in the most favorable mixtures of NaCl + $CaCl_2$.

The results afford evidence that the plasma membrane in plants is protein in character.

[5] This *Journal*, xix, p. 493, 1914.

[6] *Botanical Gazette, loc. cit.*

ON THE ESTIMATION OF FAT IN FECES.

By FRANK C. GEPHART and FRANK A. CSONKA.

(From the Russell Sage Institute of Pathology, in affiliation with the Second Medical Division of Bellevue Hospital, and from the Department of Physiology, Cornell University Medical College, New York City.)

(Received for publication, October 27, 1914.)

A great many methods have been devised for the determination of fat in feces, all of which are based upon either an extraction with a solvent, such as ether, alcohol, petroleum ether, etc., and subsequent weighing of the dried extract, or a saponification, either in alcoholic or water medium with alkali or alkaline alcoholates, separation of the free fatty acids from the soaps, and finally weighing the separated acids after drying.

The simple extraction methods can be discarded after a few considerations, the most important of which are the following:

(*a*) The material to be extracted must be dried and finely powdered or a very slow wet extraction must be resorted to, in which latter case the extract obtained will not be a true extract of the solvent employed but will contain some water-soluble material.

(*b*) A large proportion of the fatty acid complex in feces is in the form of soaps, which are either wholly or in part insoluble in the solvent.

(*c*) The solvent extracts materials other than fat, such as cholesterin, coloring matter, etc., which may constitute 50 per cent of the total extract.

(*d*) Solvents do not come in intimate contact with the material; after the extraction has proceeded for a time there is always more or less "packing."

From the above considerations it is clear that the extraction methods should be discarded and attention directed to the methods based upon a saponification. Of these there are two in general use at this time: the Liebermann-Székely method,[1] and the modification worked out by Kumagawa and Suto.[2]

[1] L. Liebermann and S. Székely: Eine neue Methode der Fettbestimmung in Futtermitteln, Fleisch, Koth etc., *Pflügers Archiv*, lxxii, pp. 360–366, 1898.

[2] Ein neues Verfahren zur quantitativen Bestimmung des Fettes und der unverseifbaren Substanzen in tierischem Material nebst der Kritik

When applied to feces the greatest source of error in the Liebermann-Székely method lies in the fact that petroleum ether extracts materials other than fatty acids, the chief sources of error being cholesterin and a small amount of other unsaponifiable material. Secondly, a single extraction without purification can yield only a crude extract; there is great danger of the presence of traces of sulphuric acid in the petroleum ether.

We have made a great many determinations of fat in feces by the Kumagawa-Suto method and find that in several stages of the process troublesome obstacles are presented. Chief among these may be mentioned the emulsions formed during the shaking out procedures, especially in the last extraction, where the fatty acid mixture is saponified and then shaken with petroleum ether to remove the unsaponifiable material. This extraction must be made in alkaline solution in the presence of soaps, so that optimum conditions are present for the formation of emulsions. Secondly, drying fatty acid mixtures at 50°C. (the initial drying of the crude ether extract) to remove moisture mechanically carried down by the ether is not a very satisfactory or rapid procedure. Finally, it often requires a day's time to secure a constant weight of the purified fatty acids in petroleum ether, and we have found in working with pure materials dissolved in petroleum ether that a constant weight can be obtained before the last traces of petroleum ether have been removed.

Inasmuch as no data are presented by Kumagawa and Suto to show the efficiency of their method when applied to pure materials, it was thought advisable to conduct such experiments. Pure tristearin and tripalmitin were saponified by the Kumagawa-Suto procedure. In spite of the constant stirring directed it was difficult to obtain a mixture; the materials persisted in floating on top of the alkali and forming a hard soap on the outer edges. Consequently when the directed time of saponification was ended, hard, compact masses were present, which could be broken only with difficulty, and which were either totally insoluble or slightly soluble in water. When the mixture was treated with acid in the separatory funnel preparatory to the ether treatment no

einiger gebrauchlichen Methoden, *Biochem. Zeitschr.*, viii, pp. 212-347, 1909; M. Kumagawa: Festbestimmung nach Kumagawa-Suto, *Abderhaldens Handbuch der biochemischen Arbeitsmethoden*, v, p. 477, 1909.

apparent change was effected. An explanation of this is not difficult. Neither the original esters nor the soaps formed are soluble in the saponifying medium. There is no intimate contact between the two, and complete saponification cannot be expected.

Frequent references are made in the literature to the presence of cholesterin esters of fatty acids in stools, and to the difficulty with which they saponify. We thought it might be advisable to saponify a cholesterin ester by the Kumagawa-Suto method. Accordingly, the cholesterin ester of stearic acid was prepared from stearyl chloride (prepared by the method of Kraft and Burger[3]) by the method followed by Abderhalden and Kautzsch.[4] Our compound melted at 83°C. and exhibited the properties described by Abderhalden and Kautzsch. Two attempts to saponify the ester by the Kumagawa-Suto method were made with the following results:

1. 1.0543 gm. ester yielded 1.0678 gm. "crude fatty acid" and 0.9245 gm. "unsaponifiable," indicating 0.1433 gm., or 13.59 per cent fatty acid. Theoretically, 1 gm. ester yields 0.4370 gm. fatty acid. Therefore, saponification was 31.10 per cent complete.
2. 1.0322 gm. ester yielded 1.0499 gm. "crude fatty acid" and 0.9331 gm. "unsaponifiable," indicating 0.1168 gm. or 11.32 per cent fatty acid. Therefore saponification was 25.31 per cent complete.

The explanation of these low results is the same as in the case of the triglycerides; the ester is insoluble in the saponifying medium. Being soluble in ether, the residual unsaponified ester is carried to the last stage in the process, where it is partially saponified.[5] If the last stage in the process, the saponification with alcoholic KOH, were repeated a number of times, no doubt higher results would be obtained, but when the procedure is followed verbatim, low results are unavoidable. The "unsaponified" residue presented the same crystalline form as the original ester crystallized from

[3] *Ber. d. deutsch. chem. Gesellsch.*, xvii, p. 1380, 1884.

[4] E. Abderhalden and K. Kautzsch: *Zeitschr. f. physiol. Chem.*, lxv, p. 75, 1910.

[5] Since the completion of our work, Thaysen (Beiträge zur physiologischen Chemie der Cholesterine und der Cholesterinestern, *Biochem. Zeitschr.*, lxii, p. 92, 1914) reports an unsuccessful saponification of cholesterin ester by the Kumagawa-Suto method, in an attempt to determine cholesterin.

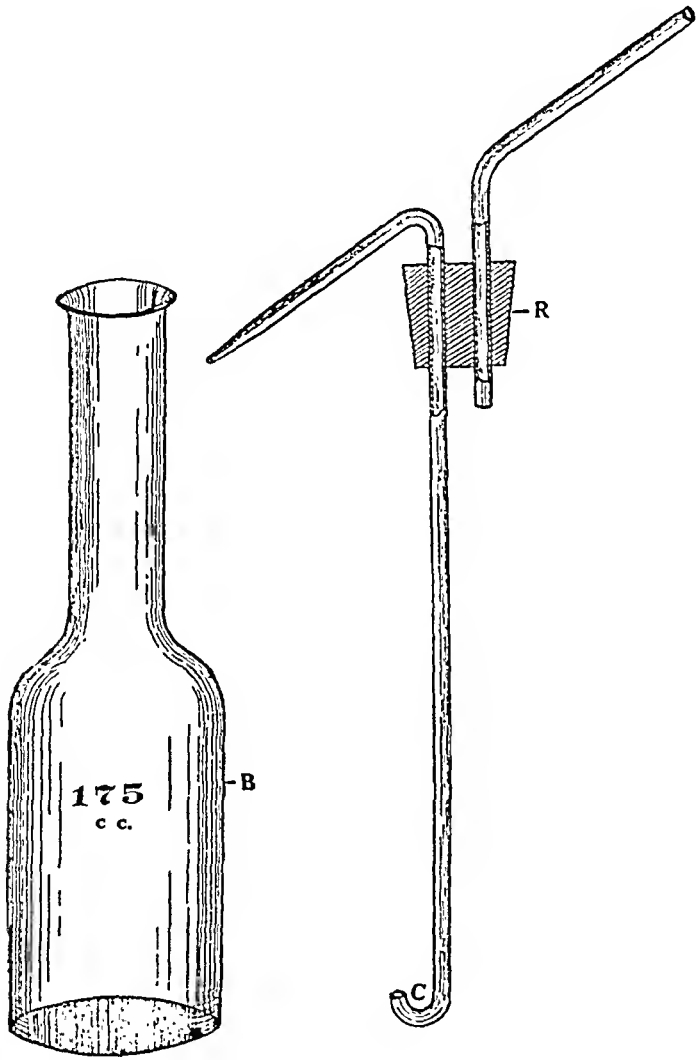
petroleum ether, and yielded additional fatty acid when subjected to our proposed method of saponification (see below). Inasmuch as the entire process up to the last stage seems to be of no avail when applied to pure materials and cholesterin esters, it was thought desirable, to work out a procedure to correct these difficulties as well as to eliminate, as far as possible, the time-consuming details alluded to above.

The procedure finally adopted is clearly a modification of the Liebermann-Székely method. We carry on the saponification in alcoholic medium with KOH, acidify, extract several times with ether, wash the combined ether extract with water until it is free of HCl and alcohol, removing in this manner the lower acids of the series if they should be present. We evaporate and dry the ether extract, take it up with petroleum ether, filter, and titrate the free fatty acids with alcoholic KOH. In this manner we estimate only the three higher fatty acids, oleic, palmitic, and stearic. On account of the presence of cholesterin and other unsaponifiable material it would be useless to evaporate the titrated solution to dryness and weigh the residual soaps. The advantage of weighing the titrated residue of soaps lies in the fact that by such a procedure the fats are determined directly, a factor being unnecessary; but, as stated above, the presence of other material vitiates the results, and removal of these interfering substances is, as already pointed out, not a satisfactory procedure. Purification and weighing are, however, unnecessary; because the molecular weights of oleic, palmitic, and stearic acids are so nearly the same (oleic, 282.3; palmitic, 256.3; stearic, 284.3) that these acids can all be estimated together by titration. The fatty acids separated from feces consist chiefly of stearic acid, hence we use the following factor: 1 cc. $\frac{N}{10}$ = 28.43 mgm. stearic acid or 29.70 mgm. tristearin. We assume in this procedure that the acidity of the purified extract is due entirely to the fatty acids referred to above. The Kumagawa-Suto procedure is based upon the same assumption, with the possible exception that the mixture obtained by Kumagawa-Suto may contain, in addition to these fatty acids, also some of the lower members of the series, as no steps are taken to remove them. The so called volatile fatty acids are quantitatively volatile when distilled with steam, but cannot be removed quantitatively by simple evaporation with

ether, as the boiling point of the lowest member of the series is over 100°C. Lactic acid, in addition to being soluble in water, is insoluble in petroleum ether, as shown by Kumagawa and Suto. It was thought that possibly the bile acids, glycocholic and taurocholic acids, might interfere, as they are present in stools, in pathological conditions, as the sodium salts. Blanks were run on sodium glycocholate and taurocholate. Ammonia was evolved during the saponification and the acids were converted into cholic acid, which was removed quantitatively in the ether extract; but when the product was dried and extracted with petroleum ether, not a trace of acidity was found. The detailed procedure which we use is the following:

Weigh out accurately 2 or 3 gm. of dried stool (finely powdered and meshed) or 5 to 7 gm. of well mixed moist stool (employ 0.5 to 1 gm. of fat) into the special form of flask shown in the cut. Add 20 cc. of 95 per cent alcohol, 4 gm. of stick potassium hydrate, and a glass bead. Immerse the flask in a boiling water bath and boil under a reflux condenser for one hour. Cool, add 50 cc. of water, and 20 cc. of 20 per cent HCl in portions of 5 cc. each, cooling the flask after each addition of the acid. Add 50 cc. of ether, insert stopper and shake well several times. Allow to stratify and repeat shaking. After the ether layer has separated, rinse back stopper and side of flask with a few drops of ether, insert wash bottle attachment and by gentle pressure blow the ether layer into a 250 cc. Squibb separatory funnel (pear shape). Repeat ether extraction twice with 50 cc. portions and finally extract with 25 cc. of ether, collecting all the ether extracts in the separatory funnel (three 50 cc. extractions and one 25 cc. extraction). If a slight emulsion persists during the extraction, allow the exit tube to dip below the emulsion and transfer it along with the ether to the separatory funnel, where it is easily broken up by gentle shaking with the excess of ether, after which it can be returned to the flask before the next extraction. Wash the combined ether extract in the separatory funnel with four 50 cc. portions of water. Combine the ether extracts and test the few drops of water collected below with litmus or silver nitrate. The crude ether extract should now be free from hydrochloric acid and lower fatty acids, as well as alcohol. Transfer the ether to a 300 cc. Erlenmeyer flask, add a glass bead, and distil off the

ether on a warm water bath, recovering the ether by the use of a condenser. Place the flask containing the residue in a vacuum desiccator, using phosphorus pentoxide as a desiccating agent. Over night, with a good vacuum, the fatty acid mixture will



become free from moisture. (The mixture should be free from ether before being placed in the desiccator, otherwise splattering sometimes occurs as the air is exhausted.) When dry, the flask is removed, and 50 cc. of light boiling petroleum ether are added.

The flask is rotated at frequent intervals and allowed to stand one hour, after which the solution is filtered through a thick plug of fat-free cotton or asbestos, with the special form of filtering tube described by Kumagawa and Suto or that of Mottram.[6] Wash the flask and filter well with petroleum ether and collect the filtrate in an Erlenmeyer flask. (The filtrate should be perfectly clear; otherwise the cotton has not been packed sufficiently.) Add a glass bead, dip the flask containing the petroleum ether solution into a warm water bath, allow to boil one minute and immediately titrate with $\frac{N}{10}$ alcoholic KOH,[7] using a few drops of alcoholic phenolphthalein as an indicator. The titration is completed when the pink color persists in the hot solution for one minute. The calculation is simple:

$$\frac{\text{cc. } \frac{N}{10} \times 0.0297}{\text{Weight of material taken}} \times 100 = \text{per cent tristearin.}$$

Remarks concerning the various steps.

We prefer to use the saponifying medium described; because it is well known that the fats and fat-containing materials are soluble in this medium and are brought into intimate contact with each other, insuring a rapid saponification. We attempted to use a prepared saturated alcoholic solution of KOH, but found that the deep color which developed upon standing was unfortunately soluble in ether and petroleum ether and interfered with the titration. We also found that the resinous aldehydes formed with long standing interfered with the determination. Even when prepared in the cold, in an amber bottle, there was a slight discoloration upon long standing. Light seems to be a greater factor in the development of color than the heat developed by the solution of the alkali. When used as described there is practically no color developed from this source, certainly not enough to interfere, and no interfering substances are formed. The ether used should be purified by being first washed with water, dried with calcium chloride, and distilled. We prefer to use phosphorus pentoxide as a desiccating agent because we have noticed that in using sulphuric acid there is a slight volatilization of the acid (approximately 0.1 cc. $\frac{N}{10}$).[8] Absolute alcohol cannot be used for drying fatty acids. We have found that when either pure stearic or palmitic acids are evaporated to dryness with an excess of absolute alcohol, taken up with petroleum ether and allowed to

[6] *Journ. of Physiol.*, xl, pp. 122–134, 1910.

[7] More dilute standard may be used when the stool has a low fat content.

[8] In this connection see Gore: this *Journal*, xv, p. 259, 1913.

stand for one hour, a turbidity forms which gradually separates as a fine white precipitate, insoluble in petroleum ether, but soluble in alcohol or ether and which titrates with alkali. The formation of this substance would lead to low results. We are inclined to believe that it is an anhydride rather than an ethyl ester.[9] It is formed in such small quantity that we have been unable to obtain enough of it for a reliable melting point determination. If the clear petroleum ether solution, which has been freed from this substance by filtration, is again evaporated with absolute alcohol, the phenomenon is repeated.

The petroleum ether used should be redistilled (40° to 60°C.) and should be free from residue and neutral in reaction.

Washing the crude ether extract with water removes whatever traces of lower fatty acids may be present as well as lactic acid, which in any event is insoluble in petroleum ether. We have found in working with butter that low results are obtained because of this fact. In order to recover the lower fatty acids from water solution with ether it is necessary to "salt out," as shown by Liebermann and Székely.

The titration under conditions described gives a sharp end-point, as one obtains in ordinary acidimetry. We have found no occasion to use phenoltetrachlorphthalein, as mentioned by Orndorff and Black,[10] or Meister alkali blue 6b, suggested by Leathes,[11] but recommend, instead, the introduction of a small amount of talcum, which serves admirably as a background, in case such is thought desirable. As is well known, there is no interfering dissociation of the soaps formed during the titration.[12]

We advise that the standard $\frac{N}{10}$ alcoholic KOH be prepared in the following way: Absolute alcohol should be used and should be free from all traces of organic impurities. Place 1 liter in an amber glass-stoppered bottle in the ice chest and when cold add 6 gm. of stick KOH with frequent shaking, allow to stand until the solution is complete and the insoluble potassium carbonate has settled. Carefully decant into another amber glass-stoppered bottle and allow the solution to assume the room temperature. It may be standardized by titration with standard hydrochloric acid, using phenolphthalein as indicator, or by the following method, which we have found quite convenient:

Weigh out approximately 1 gm. of pure cream of tartar, add water and a few drops of phenolphthalein. Run in 40 cc. of the alcoholic KOH into the flask and heat to boiling to remove carbon dioxide. Complete the titration in hot solution. One gm. of cream of tartar equals 53.14 cc. $\frac{N}{10}$. The solution being stronger than $\frac{N}{10}$ will require the use of less than 50 cc. We have found that when alcoholic KOH is prepared in this way it is colorless and retains its titer indefinitely.

---

[9] W. H. Emerson and H. N. Dumas: *Journ. Amer. Chem. Soc.*, xxxi, p. 949, 1909.

[10] W. R. Orndorff and J. A. Black: *Amer. Chem. Journ.*, xli, p. 349, 1909.

[11] J. B. Leathes: *The Fats*, p. 76, London, 1910.

[12] See A. Kanitz: *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 400, 1903.

The results obtained are the following:

1. Tristearin (Kahlbaum)

Substance taken gm.	cc. $\frac{N}{10}$	per cent
0.7220	24.30	99.93
0.6034	20.45	100.65
0.8583	29.17	100.94
1.0661	36.09	100.54

2. Tripalmitin (Kahlbaum)

0.7424	27.67	100.10
1.0991	40.84	99.92

3. Triolein (Kahlbaum)

0.7101	24.18	100.42
0.7114	24.12	99.99
0.6035	20.45	99.93
1.0143	34.47	100.22

4. Olive oil (72 per cent olein, 28 per cent palmitin) (Leach)

0.8946	30.62	98.44
0.9758	33.32	98.20

Olive oil contains 1.5 per cent unsaponifiable material.[13]

5. Whole milk: 10 cc. of milk: 12.42 cc. $\frac{N}{10}$ KOH: 3.56 per cent mixed fat. (Using a mean of olein, palmitin and stearin.) Soxhlet method: 3.63 per cent fat.

6. Modified milk: 25 cc. milk: 7.59 cc. $\frac{N}{10}$ KOH: 0.87 per cent mixed fat. Soxhlet method: 0.87 per cent fat.

7. Cholesterin ester of stearic acid.

Substance taken gm.	cc. $\frac{N}{10}$	Stearic acid per gm. gm.
1.1240	18.40	0.4650
0.9155	15.01	0.4660

Theory: 0.4370.

(Dried in sulphuric acid desiccator.)

8. Cholesterin ester of stearic acid. 0.5445 gm. ester: 8.53 cc. $\frac{N}{10}$ KOH: 0.2425 gm., or 44.54 per cent stearic acid. Theory: 43.70 per cent. (Dried in P_2O_5 desiccator.)

We were unable to make duplicates in the last determinations because our supply of ester was exhausted. When the attempt to saponify this ester in aqueous KOH was made, only a trace of fatty acid was recovered because of its insolubility in water.

The residual "unsaponifiable" material from the Kumagawa-Suto saponification of the cholesterin ester yielded in the first case 6.26 cc. $\frac{N}{10}$ or 0.1780 gm. of stearic acid, and in the second case 9.14 cc. $\frac{N}{10}$ or 0.2599 gm. of stearic acid when subjected to our procedure.

[13] *Biochem. Handlexikon*, iii, p. 97, 1910.

9. Feces.

Substance taken	cc. $\frac{N}{10}$	Stearic acid
gm.		*per cent*
1.4680	23.04	44.62
1.1066	17.80	45.74

6.5970 gm. of feces saponified and made up to 100 cc. 25 cc. portions gave the following results.

1. 26.05 cc. $\frac{N}{10}$: 44.90 per cent stearic acid.
2. 26.05 cc. $\frac{N}{10}$: 44.90 per cent stearic acid.

A comparison with the Kumagawa-Suto method is shown in the following analyses of two dry stools from typhoid patients.

I.

	SUBSTANCE	FATTY ACID	CALCULATED AS TRISTEARIN
	gm.		*per cent*
K. S.	1.6170	0.1305 gm.	8.43
Proposed method	1.1034	3.14 cc. $\frac{N}{10}$	8.45

II.

K. S.	2.5807	0.2000 gm.	8.06
Proposed method	1.8056	5.10 cc. $\frac{N}{10}$	8.38

In conclusion we wish to thank Mr. Rudoph H. Harries for assistance in carrying out much of the analytical work.

RÉSUMÉ.

1. By the Liebermann-Székely method one determines a small amount of cholesterin with the fatty acids.

2. The Kumagawa-Suto method is laborious and difficult of manipulation, and does not yield good results with pure fats or cholesterin esters.

3. The proposed method is time-saving, and yields good results with pure fats, cholesterin esters, and feces.

The steps of the proposed method may be summarized as follows:

a. Weigh out finely powdered or well mixed moist sample.

b. Saponify with KOH in alcohol (4 gm. of stick KOH and 20 cc. of 95 per cent alcohol).

c. Dilute with 50 cc. of water and acidify with HCl (20 cc. of 20 per cent in 5 cc. portions).

d. Shake out with ether and wash ether extract.

e. Distil off ether and dry fatty acids.

f. Take up with petroleum ether, filter, and titrate with $\frac{N}{10}$ alcoholic KOH.

g. Calculation:

$$\frac{\text{cc. } \frac{N}{10} \times 0.0297}{\text{Weight of substance taken}} \times 100 = \text{per cent tristearin.}$$

INDEX TO VOLUME XIX.

Acidity of fresh milk of cows, and a method for the determination of acidity, 73.

Acidosis in omnivora and herbivora and its relation to protein storage, 399.

Acids and salts, antagonism between, 517.

Albumen, coagulation of, by pressure, 511.

Alcohol, inhibition of autolysis by, 57.

Alfalfa hay and corn grain, comparative efficiency of nitrogen of, for milk production, 127.

ALSBERG, CARL L.: Note on the proteins of the blood of *Limulus polyphemus* L., 77; —— and WILLIAM MANSFIELD CLARK: The solubility of oxygen in the serum of *Limulus polyphemus* L. and in solutions of pure *Limulus* haemocyanin, 503.

Amino-acid mixtures. Metabolic changes in muscular tissues. I, 229.

Anticoagulating effect of hirudin, 305.

Arsenic, distribution of, in a human body, 513.

Aspergillus niger, a new polysaccharide, mycogalactan, in, 235; *terricola*, enzymes of, 459.

Autolysis, inhibition of, by alcohol, 57; of *Glomerella*, formation of hexone and purine bases in, 257.

Benzidine test for occult blood, some factors involved in, 445.

BERGEIM, OLAF, MARTIN E. REHFUSS and PHILIP B. HAWK: Gastro-intestinal studies III (studies on water drinking XXI). Direct demonstration of the stimulatory power of water in the human stomach, 345.

BLATHERWICK, NORMAN R.: See Underhill and Blatherwick, 39, 119.

Blood, catalytic reactions of. I. A study of some of the factors involved in the benzidine test for occult blood, 445; fat content of, 1; normal human, urea and total non-protein nitrogen in, 31; proteins of, of *Limulus polyphemus* L., 77; sugar content of, after thyreoparathyroidectomy, influence of subcutaneous injections of dextrose and calcium lactate on, 119; sugar, determination of, 285; sugar of dog, normal level of, 297; variations in fat content of, under approximately normal conditions, 1.

BLOOR, W. R.: Studies on blood fat. I. Variations in the fat content of the blood under approximately normal conditions, 1.

BOSWORTH, ALFRED W.: The action of rennin on casein, 397; —— and LUCIUS L. VAN SLYKE: The phosphorus content of casein, 67; ——, see Van Slyke and Bosworth, 73.

BRAMAN, WINFRED W.: A study in drying urine for chemical analysis, 105.

BRIDGMAN, P. W.: The coagulation of albumen by pressure, 511.
Butter fat, isolation of the substance in, which exerts a stimulating influence on growth, 245.

CALDWELL, GEORGE T. and H. GIDEON WELLS: The purine enzymes of the opossum (*Didelphis virginiana*), 279; see also Wells and Caldwell, 57.
Carbohydrate metabolism, studies in. III, 119.
Casein, action of rennin on, 397; phosphorus content of, 67.
Catalytic reactions of blood, 445.
Cereal grains and milk, proteins of, value of, for growth in the pig, 323.
CLARK, ELIZABETH E.: See Tracy and Clark, 115.
CLARK, WILLIAM MANSFIELD: See Alsberg and Clark, 503.
Coagulation of albumen by pressure, 511.
Corn grain and alfalfa hay, comparative efficiency of nitrogen of, for milk production, 127; or wheat grain, influence on growth of rations restricted to, 373.
Corpus luteum substance, effects of, upon ovulation in the fowl, 263.
CRAWFORD, ALBERT C. and WALTER K. WATANABE: Parahydroxyphenylethylamine, a pressor compound in an American mistletoe, 303.
Creatine-free diet, prolonged, excretion of creatinine by human individuals on, 487.
Creatinine elimination and basal metabolism, 239; excretion by human individuals on a prolonged creatine-free diet, 487; excretion by normal women, 115.
CSONKA, FRANK A.: See Gephart and Csonka, 521.
CULLEN, GLENN E.: See Van Slyke and Cullen, 141, 211.
CURTMAN, L. J.: See Lyle, Curtman and Marshall, 445.

DAVIS, MARGUERITE: See McCollum and Davis, 245.
Determination of acidity of fresh cows' milk, method for, 73; of fat in feces, 521; of iodine in connection with studies in thyroid activity, 251; of sugar in blood, 285; of urea, 211.
Dextrose, influence of subcutaneous injections of, upon blood sugar content and tetany after thyreoparathyroidectomy, 119.
2,8-dioxy-9-ethylpurine, 25.
Diuresis, effect of, on milk secretion, 127.
DOX, ARTHUR W. and RAY E. NEIDIG: The soluble polysaccharides of lower fungi. II. Mycogalactan, a new polysaccharide in *Aspergillus niger*, 235.
DUBIN, H.: See Raiziss, Dubin and Ringer, 473.

Endogenous uric acid metabolism, 473.
Enzymes, action of, further studies on, 181; in general, mode of action of, 141; of *Aspergillus terricola*, 459; purine, of opossum, 279.

Fat content of blood, 1; content of blood, variations in, under approximately normal conditions, 1; in feces, 521; of butter, isolation of the substance in, which exerts a stimulating influence on growth, 245.
Feces, fat in, 521.
Fungi, lower, soluble polysaccharides of, 235.

GAMBLE, JAMES L.: See Palmer, Means and Gamble, 239.
Gastro-intestinal studies III (studies on water drinking XXI), 345.
GEPHART, FRANK C. and FRANK A. CSONKA: On the estimation of fat in feces, 521.
Glomerella, formation of hexone and purine bases in autolysis of, 257.
GOLDSCHMIDT, SAMUEL: The metabolism of an isomer of xanthine and some isomers of the methylxanthines, 83.
Growth in the pig, value of proteins of cereal grains and of milk for, and influence of plane of protein intake on, 323; influence on, of rations restricted to the corn or wheat grain, 373; isolation of the substance in butter fat which exerts a stimulating influence on, 245.

Haemocyanin, pure, of *Limulus*, solubility of oxygen in solutions of, 503.
HART, E. B. and G. C. HUMPHREY: The comparative efficiency for milk production of the nitrogen of alfalfa hay and the corn grain. (Preliminary observations on the effect of diuresis on milk secretion), 127; —— and E. V. McCOLLUM: Influence on growth of rations restricted to the corn or wheat grain, 373; ——, see Steenbock, Nelson and Hart, 399.
HAWK, PHILIP B.: See Bergeim, Rehfuss and Hawk, 345.
HENDRIX, BYRON M.: See Johns and Hendrix, 25.
Hexone bases, formation of, in autolysis of *Glomerella*, 257.
Hirudin, immunization against the anticoagulating effect of, 305.
HUMPHREY, G. C.: See Hart and Humphrey, 127.
Hydrogen ion concentration, effect of, on urease, 181.

Immunization against the anticoagulating effect of hirudin, 305.
Inhibiting substances, effect of, on urease, 181.
Inhibition of autolysis by alcohol, 57.
Iodine determination in connection with studies in thyroid activity, 251.
Ions, oppositely charged, and antagonistic action of salts, 431.

JOHNS, CARL O. and BYRON M. HENDRIX: Researches on purines. XV. On 2-oxy-5-amino-6-ethylaminopyrimidine and 2, 8-dioxy-9-ethylpurine, 25.

KENDALL, E. C.: The determination of iodine in connection with studies in thyroid activity, 251.

Lactate, calcium, influence of subcutaneous injections of, upon blood sugar content and tetany after thyreoparathyroidectomy, 119.
LAMB, A. R.: See Hart and Humphrey, 127.
Limulus polyphemus L., proteins of the blood of, 77; solubility of oxygen in serum of, 503.
LOEB, JACQUES: Is the antagonistic action of salts due to oppositely charged ions?, 431.
LOEB, LEO: See Vera and Loeb, 305.
LYLE, W. G., L. J. CURTMAN and J. T. W. MARSHALL: The catalytic reactions of blood. I. A study of some of the factors involved in the benzidine test for occult blood, 445.

MARSHALL, J. T. W.: See Lyle, Curtman and Marshall, 445.
MATTHEWS, S. A. and C. FERDINAND NELSON: Metabolic changes in muscular tissue, I. The fate of amino-acid mixtures, 229.
McCOLLUM, E. V.: The value of the proteins of the cereal grains and of milk for growth in the pig, and the influence of the plane of protein intake on growth, 323; —— and MARGUERITE DAVIS: Observations on the isolation of the substance in butter fat which exerts a stimulating influence on growth, 245; ——, see Hart and McCollum, 373.
McLEAN, FRANKLIN C. and LAURENCE SELLING: Urea and total non-protein nitrogen in normal human blood: Relation of their concentration to rate of elimination, 31.
MEANS, JAMES H.: See Palmer, Means and Gamble, 239.
Metabolic changes in muscular tissue, 229.
Metabolism, basal, and creatinine elimination, 239; carbohydrate, 119; endogenous, of uric acid, 473; of an isomer of xanthine and some isomers of the methylxanthines, 83.
Methylxanthines, metabolism of some isomers of, 83.
Milk and cereal grains, value of, for growth in the pig, 323; fresh, of cows, acidity of, 73; production, comparative efficiency for, of nitrogen of alfalfa hay and corn grain, 127; secretion, effect of diuresis on, 127.
Mistletoe, American, parahydroxyphenylethylamine, a pressor compound in, 303.
MORSE, MAX: The effective p ciple in thyroid accelerati involution in frog larvae, 421
Muscular tissue, metabolic chang in, 229.
Mycogalactan, a new polysacch ride in *Aspergillus niger*, 235

NEIDIG, RAY E.: See Dox and Ne dig, 235.
NELSON, C. FERDINAND: See Ma thews and Nelson, 229.
NELSON, V. E.: See Steenbock, Ne son and Hart, 399.
Nitrogen of alfalfa hay and co grain, comparative efficien of, for milk production, total non-protein, in no human blood, 31.
Non-protein nitrogen, total, in no mal human blood, 31.

Opossum (*Didelphis virginian* purine enzymes of, 279.
OSTERHOUT, W. J. V.: The effec alkali on permeability, 335; effect of acid on permeabili 493; Antagonism between acid and salts, 517.
Ovulation in the fowl, effect corpus luteum substance up 263.
2-oxy-5-amino-6-ethylaminopyri dine, 25.
Oxygen, solubility of, in the ser of *Limulus polyphemus* L. in solutions of pure *Limu* haemocyanin, 503.

PALMER, WALTER W., JAMES MEANS and JAMES L. GAMB Basal metabolism and cre nine elimination, 239.
Parahydroxyphenylethylamine, pressor compound in Americ mistletoe, 303.

ARL, RAYMOND and FRANK M. SURFACE: Studies on the physiology of reproduction in the domestic fowl. IX. On the effect of corpus luteum substance upon ovulation in the fowl, 263.

rmeability, effect of acid on, 493; effect of alkali on, 335.

enolsulphonephthalein, elimination of, in acute and chronic tartrate nephritis, 39.

osphorus content of casein, 67.

lysaccharides, soluble, of lower fungi. II. Mycogalactan, a new polysaccharide in *Aspergillus niger*, 235.

ssor compound in American mistletoe, parahydroxyphenylethylamine, 303.

tein intake, influence of, on growth in the pig, 323; storage in relation to acidosis in omnivora and herbivora, 399.

oteins of cereal grains and of milk, value of, for growth in the pig, 323; of the blood of *Limulus polyphemus* L., 77.

rine bases, formation of, in autolysis of *Glomerella*, 257; enzymes of opossum, 279.

nes, researches on. XV, 25.

IZISS, G. W., H. DUBIN and A. I. RINGER: Studies in endogenous uric acid metabolism, 473; ——, see Ringer and Raiziss, 487.

ED, HOWARD S.: The formation of hexone and purine bases in the autolysis of *Glomerella*, 257.

HFUSS, MARTIN E.: See Bergeim, Rehfuss and Hawk, 345.

anin, action of, on casein, 397.

eproduction in the domestic fowl, studies on the physiology of. IX, 263.

RINGER, A. I. and G. W. RAIZISS: The excretion of creatinine by human individuals on a prolonged creatine-free diet, 487; see also Raiziss, Dubin and Ringer, 473.

Salts and acids, antagonism between, 517; antagonistic action of, due to oppositely charged ions, 431.

SCALES, F. M.: The enzymes of *Aspergillus terricola*, 459.

SCHAFFER, PHILIP A.: On the determination of sugar in blood, 285; On the normal level of blood-sugar in the dog, 297.

SELLING, LAURENCE: See McLean and Selling, 31.

STEENBOCK, H., V. E. NELSON and E. B. HART: Acidosis in omnivora and herbivora and its relation to protein storage, 399.

Stomach, human, stimulatory power of water in, 345.

Sugar in blood, determination of, 285; in blood, influence of subcutaneous injections of dextrose and calcium lactate on, after thyreoparathyroidectomy, 119; in blood of dog, normal level of, 297.

SURFACE, FRANK M.: See Pearl and Surface, 263.

Tartrate nephritis, chronic and acute, elimination of phenolsulphonephthalein in, 39.

Tetany after thyreoparathyroidectomy, influence of subcutaneous injections of dextrose and calcium lactate on, 119.

Thyreoparathyroidectomy, influence of subcutaneous injections of dextrose and calcium lactate on blood sugar content and tetany after, 119.

Thyroid activity, iodine determination in connection with studies in, 251; effective principle in, accelerating involution in frog larvae, 421.
Tissue, muscular, metabolic changes in, 229.
TRACY, MARTHA, and ELIZABETH E. CLARK: The excretion of creatinine by normal women, 115.

UNDERHILL, FRANK P.: The distribution of arsenic in a human body, 513; —— and NORMAN R. BLATHERWICK: The elimination of phenolsulphonephthalein in acute and chronic tartrate nephritis, 39; Studies in carbohydrate metabolism. VII. The influence of subcutaneous injections of dextrose and of calcium lactate upon the blood sugar content and upon tetany after thyreoparathyroidectomy, 119.
Urea, determination of, use of a permanent preparation of urease in, 211; in normal human blood, 31.
Urease, effect of hydrogen ion concentration and of inhibiting substances on, 181; mode of action of, 141; permanent preparation of, and its use in the determination of urea, 211.
Uric acid metabolism, endogenous, 473.
Urine, drying of, for chemical analysis, 105.

VAN SLYKE, DONALD D. and GLENN E. CULLEN: The mode of action of urease and of enzymes in general, 141; A permanent preparation of urease and its use in the determination of urea, 21[illegible]
—— and GOTTHARD ZACHARIAS: The effect of hydrogen ion concentration and of inhibiting substances on urease. Further study on the mode of enzyme action, 181.
VAN SLYKE, LUCIUS L. and ALB[illegible] W. BOSWORTH: The causes of acidity of fresh milk of cows and a method for the determination of acidity, 73; see Bosworth and Van Slyke, [illegible]
VERA, MIGUEL and LEO L[illegible] [illegible]munization against the anti-coagulating effect of [illegible] 305.

WATANABE, WALTER K.: See Crawford and Watanabe, 303.
Water drinking, studies on, XXI Gastro-intestinal studies III 345; in the human stomach stimulatory power of, 345.
WELLS, H. GIDEON and GEORGE T. CALDWELL: The inhibition of autolysis by alcohol, 57; see also Caldwell and Wells, 279.
Wheat or corn grain, influence on growth of rations restricted to, 373.
WILLAMAN, J. J.: See Hart and Humphrey, 127.

Xanthine, metabolism of an isomer of, 83.

ZACHARIAS, GOTTHARD: See Van Slyke and Zacharias, 181.